Validation and clinical evaluation of a novel method to measure miltefosine in leishmaniasis patients using dried blood spot sample collection

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ABSTRACT

To facilitate future pharmacokinetic studies of combination treatments against leishmaniasis in remote endemic regions, a simple and cheap sampling methodology was required for miltefosine quantification. The aim of this study was to validate a liquid chromatography-tandem mass spectrometry method to quantify miltefosine in dried blood spots (DBS) and to validate its use in Ethiopian visceral leishmaniasis (VL) patients. Since hematocrit (Ht) values are typically severely decreased in VL patients, regressing to normal during treatment, the method was evaluated over a range of clinically relevant Ht values.

Miltefosine was extracted from DBS using a simple pre-treatment method with methanol, resulting in >97% recovery. The method was validated over a calibration range of 10-2,000 ng/mL and accuracy and precision were within ±11.2% and ≤7.0% (≤19.1% at LLOQ), respectively. The method was accurate and precise for blood spot volumes between 10−30 µL and for an Ht of 20−35%, though a linear effect of Ht on miltefosine quantification was observed in the bioanalytical validation. DBS samples were stable for at least 162 days at 37°C.

Clinical validation of the method using paired DBS and plasma samples from 16 VL patients showed a median observed DBS:plasma miltefosine concentration ratio of 0.99, with good correlation (Pearson's r=0.946). Correcting for patient-specific Ht did not further improve the concordance between the sampling methods.

This successfully validated method to quantify miltefosine in DBS was demonstrated to be a valid and practical alternative to venous blood sampling which can be applied in future miltefosine pharmacokinetic studies in leishmaniasis patients, without Ht-correction.

INTRODUCTION

Miltefosine is currently the only oral drug against both cutaneous (CL) and visceral leishmaniasis (VL) and new studies to evaluate the use of miltefosine-based combination therapies in VL and HIV co-infected VL patients are underway (1). Recently it was discovered that miltefosine treatment failure is associated with lower drug exposure: the time that miltefosine plasma concentrations were above 10x the 50% effective concentration (17.9 µg/mL) was correlated with final treatment failure or success (2). This stresses the need for adequate pharmacokinetic (PK) monitoring in these clinical trials.

Both CL and VL are poverty-related diseases which mainly affect populations in resource-poor and remote regions of Africa, Asia and South America. Classically, human blood plasma is collected by venous sampling for the measurement of drug concentrations, e.g. employing liquid chromatography-tandem mass spectrometry (LC-MS/MS). A bioanalytical method to quantify miltefosine in plasma has been validated and reported previously (3). However, technologies such as LC-MS/MS are not available in the regions where VL is endemic and samples therefore need to be transported to appropriate facilities for analysis. The required cold storage (3) and transport of these plasma samples is logistically highly challenging and, on top of that, expensive. In addition, plasma sampling by venipuncture is an invasive and risky sampling method, particularly for severely weakened and anaemic HIV co-infected VL patients. A large proportion of VL patients in East Africa is pediatric (4), which limits both the total volume and number of plasma PK samples that can be taken by venous blood sampling. Dried blood spot (DBS) sampling is therefore an attractive alternative to plasma sampling in these settings because it is minimally-invasive and requires only a small volume of blood (5–9), which is particularly advantageous in pediatric studies (10, 11). Additionally, storage and shipment is possible at room temperature and is therefore simple, low cost and hence is preferred in remote areas without proper laboratory setup.

A major hurdle in the application of DBS sample collection is, however, the effect of hematocrit (Ht) and blood spot volume on the measured drug concentrations (12–14). Ethiopian VL patients had a decreased median Ht of 25% (range Ht 23-30%) at initiation of treatment (15), which slowly regressed towards Ht 33% (range Ht 27-37%) after a 30 day treatment with sodium antimony

gluconate (15). HIV co-infected VL patients show similar Ht values during active VL infection (mean haemoglobin concentration of 9 g/dl, corresponding to an Ht value of approximately 27% (16)). Since miltefosine has a long terminal half-life (30.9 days) (17) and accumulates during treatment, pharmacokinetic sampling is typically performed at various time points during treatment and up to several months after end of treatment. Ht values show high within-subject variability within this period and may influence the outcome of drug measurements when using DBS sample collection.

Additionally, blood spot volume can vary widely between patients due to the variation in blood flow and the penetration of the lancet in the finger. Viscosity of the blood increases with the increase of Ht (18) and therefore the blood flow and possibly blood spot volume can be expected to be larger for patients with a lower Ht.

Here we describe the development and validation of a rapid LC-MS/MS method to quantify miltefosine levels in DBS in a range from 10-2,000 ng/mL, according to the current Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines (19, 20) and European Bioanalysis Forum (EBF) recommendations (21, 22) for DBS assays. Furthermore, this study evaluates and validates the clinical applicability of this method by comparing paired DBS and plasma samples from 16 Ethiopian HIV co-infected VL patients, who received miltefosine treatment.

METHODS

1. Chemicals and reagents

Miltefosine was purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Deuterated miltefosine (miltefosine-D4, see Figure 1), was purchased from Alsachim (Illkirch Graffenstaden, France). Methanol and water were obtained from Biosolve Ltd. (Valkenswaard, the Netherlands). Ammonia 25% was purchased from Merck (Amsterdam, the Netherlands).

2. Materials

For the collection of DBS, pure cellulose-based cards (Whatman® 903 protein saver cards) were used. These cards, together with foil bags and desiccant packages for storage of DBS were purchased from GE Healthcare Europe GmbH (Diegem, Belgium). A Harris 3.0 mm micro-punch® was used for

111 punching the DBS. Whole blood (WB) was collected in K_2EDTA BD Vacutainers® from healthy
112 volunteers and stored at 2-8°C for a maximum of 2 days. WB was adjusted to an Ht level of 30±1%
113 (Ht30 WB) to mimic the Ht of VL patients, by dilution with plasma. Ht levels were determined with
114 the Cell Dyn Hematology analyser (Abbot Diagnostics, Lake Forest, IL, USA).
115
116 **3. Preparation of calibration standards and QC samples**
117 Stock solutions of 1 mg/mL miltefosine were prepared from independent weightings in methanol-
118 water (1:1, v/v). Separate stocks were diluted to working solutions with methanol-water (1:1, v/v) for
119 the preparation of calibration standards and quality control (QC) samples. A stock solution of 1
120 mg/mL deuterated miltefosine (miltefosine-D4) was prepared and diluted to internal standard working
121 solution of 4,000 ng/mL miltefosine-D4 in methanol-water (1:1, v/v). This working solution was
122 further diluted with methanol to a 20 ng/mL miltefosine-D4 extraction solution. Stock and working
123 solutions were stored at nominally -20°C.
124 Calibration standards were diluted 1:20 (v/v) in Ht30 WB to final concentrations of 10, 20,
125 100, 500, 1,000, 1,400, 1,800 and 2,000 ng/mL. QC samples were diluted 1:20 (v/v) in Ht30 WB to
126 final concentrations of nominally 10, 24, 300 and 1,600 ng/mL (lower limit of quantification, LLOQ;
127 low level, QCL; mid level, QCM; high level, QCH, respectively). Additionally an >ULOQ (above
128 upper limit of quantification) sample of 40,000 ng/mL was prepared and used to determine dilution
129 integrity.
130 A volume of 20 µL of spiked whole blood was spotted on Whatman 903 cards and air dried at
131 least for 3 hours at room temperature. When comparing samples that were dried for 3 hours to samples
132 that were dried overnight (15-20 hours), no effect was found for the additional drying time (bias
133 within ±6.1%).
134
135 **4. Sample pre-treatment**
136 After drying, a 3.0 mm punch was taken from the center of the DBS and transferred to a 1.5 mL
137 Eppendorf tube. To prevent spot-to-spot puncher carry-over, an unspotted filter punch was taken after
138 each sample punch. 150 µL of extraction solution (20 ng/mL miltefosine-D4 in methanol) was added

to the sample with the exception of double blanks to which 150 µL of methanol was added. The tubes were mixed for 10 seconds, sonicated for 30 minutes and mixed for another 30 seconds. Subsequently the final extract was transferred to an autosampler vial and 10 µL was injected onto the HPLC column. No additional recovery of miltefosine from the blood spot was found when using longer sonication times.

5. Liquid chromatography-tandem mass spectrometry

Chromatographic separation was performed as described for the miltefosine plasma method previously validated (3), using a Gemini C18 pre-column (4.0 mm x 2.0 mm I.D.; Phenomenex, Torrance, CA, USA) and an analytical column (Gemini C18, 150 mm x 2.0 mm I.D., 5 µm particle size; Phenomenex), with an isocratic eluent of 10 mM ammonia in 95% methanol (v/v) at 0.3 mL/min. The HPLC system (Agilent 1100 series, Palo Alto, CA, USA) consisted of a binary pump, in-line degasser, autosampler (at 4°C) and column oven (at 25°C). The miltefosine concentrations were analyzed on an API-3000 triple-quadrupole mass spectrometer equipped with a turbo-ionspray source (Sciex, Framingham, MA, USA), operating in positive ion mode. Table *1* summarises the MS operating parameters.

6. Validation of assay for the quantification of miltefosine in DBS

The validation of the assay was performed according to the most current EMA and FDA guidelines for the validation of bioanalytical assays (19, 20) regarding the following aspects: calibration model, accuracy and precision, LLOQ, selectivity (endogenous interferences and cross analyte interferences), carry over (instrumentation and spot-to-spot carry-over), dilution integrity, matrix effect and recovery. Additional experiments were performed for the application of dried blood spots as a matrix according to EBF recommendations (21, 22): blood spot volume, blood spot homogeneity and different WB Ht values were tested for their effect on accuracy and precision at two concentration levels (QCL and QCH). Stability was tested up to 162 days at four nominal temperatures: -70°C, -20°C, room temperature (20-25°C) and 37°C.

7. Clinical application

As part of a larger randomized clinical trial (NCT02011958) investigating the treatment of Ethiopian HIV co-infected VL patients with high dose liposomal amphotericin B alone (40 mg/kg total dose given over 24 days) or liposomal amphotericin B (30 mg/kg total dose given over 11 days) in combination with a 28-day miltefosine regimen (2.5 mg/kg daily), paired plasma and DBS samples were collected from 16 patients. Ethical approval was obtained from the Ethiopian National Research Ethics Review Committee, the Institutional Review Board of University of Gondar in Ethiopia, and ethics committees from Médecins Sans Frontières, London School of Hygiene & Tropical Medicine and the Institute of Tropical Medicine Antwerps. Regulatory approval was obtained from the Food, Medicine and Health Administration and Control Authority in Ethiopia. All patients provided written informed consent before entering the study. DBS and plasma samples were collected simultaneously at day 29 of miltefosine treatment, one day after the last miltefosine dose, when patients are considered to have reached steady-state/maximal levels.

Plasma samples were collected using K_2EDTA BD Vacutainers® and after centrifugation, plasma was isolated and stored and transported at -20°C until analysis. DBS samples were collected from a finger-prick using a lancet (GST corporation, New Delhi, India). A drop of blood was applied on a Whatman® 903 protein saver card without touching of the filter paper with the finger tip. DBS samples were allowed to air-dry for at least 3 hours before being stored in an air- and watertight ziplock bag containing at least three desiccant packages. DBS samples were stored and transported by courier at room temperature. Ht levels of the patients were determined on a Beckman Coulter AcT Diff Hematology Analyzer (Beckman Coulter, Fullerton, CA, USA).

Observed DBS and plasma concentrations were compared using weighted Deming regression and a Bland-Altman difference plot was used to depict the agreement between both methods. All statistical analyses were performed with R (version 3.1.2). The acceptance criteria for the agreement between the observed and derived plasma concentration were based on the guideline for incurred sample reanalysis of the EMA: the difference between the observed and derived miltefosine plasma concentration should be within ±20% for at least 67% of the samples (19).

RESULTS

1. Calibration model

Calibration standards at eight concentration levels in the range from 10-2,000 ng/mL were prepared and analyzed in duplicate on three separate days at the beginning and end of the analytical run. To obtain the lowest total bias across the range, the linear regression of the analyte/internal standard peak area ratio (AR) versus the concentration of miltefosine (x) was weighted, $1/x^2$. The calibration curve was accepted if 75% of the non-zero calibration standards were within ±15% of their nominal concentration (±20% for the LLOQ). At least one calibration standard at the LLOQ and ULOQ should be accepted. All three calibration curves met these criteria and had correlation coefficients (R^2) of ≥0.9964.

2. Accuracy and precision

The accuracy and precision of the method were determined by analyzing the LLOQ, QCL, QCM and QCH in five-fold in three separate analytical runs. Intra-assay and inter-assay bias values were within ±15% of the nominal concentrations for all QC samples. As displayed in Table *2*, intra- and inter-assay precision (expressed as coefficient of variation, CV) were ≤7.0% for QCL, QCM and QCH and ≤19.1% for the LLOQ. Both the accuracy and precision of the method were therefore found to be acceptable.

3. Lower limit of quantitation

The first blank and the five LLOQ quality control samples were used to determine the signal to noise ratio in three analytical runs. The signal to noise ratio of miltefosine at LLOQ level was above 5 for all three runs (9.6, 5.3 and 5.8, respectively). Figure *1* shows representative LC-MS/MS ion chromatograms of miltefosine and the internal standard in a double blank sample and an LLOQ sample.

4. Specificity and selectivity

Six different batches of human WB were collected from six healthy donors, adjusted to Ht30 WB and both a double blank and an LLOQ sample were prepared from each batch. The samples were processed and analyzed as described above. The six LLOQ samples were all within ±20% of their nominal values. For the double blank samples, five out of six batches showed no interferences at the retention time of miltefosine over 20% of the peak area of the LLOQ sample and none of the double blanks showed a peak for miltefosine-D4 higher than 5% of the internal standard peak area. Selectivity was therefore considered sufficient.

To test the cross-analyte interference, an ULOQ sample was prepared as described above, but subsequently processed by adding methanol as the extraction solvent (excluding internal standard). Additionally, the internal standard was spiked separately to a double blank sample at the nominal concentration. No internal standard interferences were observed on the analyte signal and additionally no interference from the analyte was measured for the specific mass transition of the internal standard.

5. Dilution integrity

The mean miltefosine concentration at the end of a 28-day treatment (150 mg/day) was found to be around 30,000 ng/mL in Dutch CL patients (17). Therefore, an >ULOQ sample of 40,000 ng/mL was used in the dilution integrity experiment. The >ULOQ sample was prepared as described previously and the final extract was subsequently diluted 100 times with the final extract of a processed blank DBS (extracted with extraction solvent containing the internal standard). The dilution steps were as follows: first, 10 µL >ULOQ final extract was diluted with 90 µL of blank final extract and subsequently 10 µL of this dilution was further diluted with another 90 µL of blank final extract. The % deviation of the diluted >ULOQ samples were within ±3.3% of the nominal concentration and the precision ≤2.0% and therefore it was concluded that samples exceeding the ULOQ (up to 40,000 ng/mL) can be diluted as described, applying a dilution factor of 100.

247 **6. Carry-over**

248 Two types of carry-over are important to investigate in the validation of dried blood spots methods:
249 instrument carry-over and spot-to-spot carry-over caused by the punching device. These two sources
250 of carry-over were tested. Spot-to-spot carry-over samples were prepared by punching spots in
251 following sequence: an ULOQ sample, unspotted filter paper (to get rid of most of the carry-over), a
252 blank spot, unspotted filter paper, a blank spot. These two blank spots were processed as described
253 previously and injected after the ULOQ. The combined instrument and spot-to-spot carry-over of the
254 two samples was compared to the mean value of five LLOQ samples measured and was found to be
255 below 19.3% of the LLOQ.

256 However, in clinical practice miltefosine concentrations are often expected to exceed the
257 calibration range of 10-2,000 ng/mL. Samples with an expected concentration around the ULOQ or
258 >ULOQ should preferably be analyzed in one batch. After punching a 40,000 ng/mL >ULOQ sample,
259 carry-over is acceptable (<20% of LLOQ) at the fourth blank spot punched subsequently.

260

261 **7. Matrix factor and recovery**

262 The matrix factor (MF) and recovery were tested in six different batches of Ht30 WB, spiked at QCL
263 and QCH in singlicate. 10 µL spots were prepared from these solutions, so-called "processed DBS
264 samples". For the analysis, the entire spot was cut out and processed as described previously with 150
265 µL extraction solvent. Additionally, "matrix absent" and "matrix present" samples were prepared, for
266 which two neat solutions were first prepared: MF-L (MF-low, 24.4 ng/mL miltefosine) and MF-H
267 (MF-high, 1630 ng/mL miltefosine) in extraction solvent (20 ng/mL miltefosine-D4 in methanol). The
268 "matrix absent" samples were prepared by diluting 10 µL of these neat solutions with 140 µL of
269 extraction solvent. The "matrix present" samples were prepared by cutting out the entire 10 µL blank
270 spots of the six different Ht30 WB batches, after which 10 µL of MF-L or MF-H solution and 140 µL
271 extraction solvent was added.

272 MF was calculated for each batch by calculating the ratio of the miltefosine peak area in the
273 "matrix present" sample compared to the "matrix absent" sample. The MF at both tested
274 concentrations was around 0.3 as a result of matrix effects (ion suppression). The IS-normalized MF

was around 1.0 which indicated that the stable isotope-labeled internal standard is effectively compensating for any matrix effects. At both tested QC levels, the CV of the absolute and IS-normalised MF calculated from the six different Ht30 WB batches, were below 11.5%.

Given that the internal standard is added as extraction solution, it is not part of the sample pre-treatment and therefore the IS-normalised values were used to determine recovery. The sample pre-treatment recovery was calculated by comparing the area ratio (AR) of the "processed DBS sample" with the AR of the "matrix present" samples. IS-normalised sample pre-treatment recovery was around 100% (97.2% for QCL and 103% for QCH). At both tested QC levels the CV of the IS-normalised recovery from the 6 batches was below 6.7%.

Both the matrix effect and recovery experiments were considered acceptable because the CV calculated from the six different Ht30 WB batches was consistent and below 15%.

8. Stability

DBS QC samples were prepared at two concentration levels (QCL and QCH) as described previously and air-dried at room temperature overnight. The subsequent day the samples were stored in sealed aluminium bags with three desiccant packages at four temperatures: -70°C, -20°C, room temperature (20-25°C) and 37°C. Stability was tested at day 34, 58, 107 and 162 and the measured concentration was within ±12.5% of the nominal concentration and precision was ≤10.7%. Stability of miltefosine in DBS was proven to be at least 5 months (162 days) at temperatures ranging from -70°C to 37°C, when stored in sealed aluminium bags with three desiccant packages.

9. Blood spot homogeneity

Blood spot homogeneity was investigated in 20 µL Ht30 WB DBS at QCL and QCH level in triplicate. 3.0 mm punches were taken at the perimeter instead of the center of the spot. The bias was 21.8% for the QCL and 18.0% for the QCH (CV ≤ 7.4%), which points out the importance of punching the center of the spot.

10. Effect of blood spot volume

For all of the validation procedures described here, a standard fixed spot volume of 20 µL was used. QCL and QCH were spotted in blood spot volumes reflecting the procedure in clinical practice: 10, 15, 25 and 30 µL. Samples were analyzed in triplicate. Accuracy and precision were all within ±13.4%, indicating that variability in the blood spot volume between 10 to 30 µL has no effect on the accuracy and precision of the method [data not shown].

11. Effect of hematocrit

Human WB was adjusted to a range of Ht values that were expected in clinical practice in HIV co-infected VL patients: 20%, 23%, 31% and 35%. For each Ht level, QCL and QCH samples were spiked and analyzed in triplicate. The accuracy and precision of DBS samples within this Ht range were all within ±14.1% and ≤7.2% respectively and were therefore considered acceptable (within ±15%) [data not shown]. However, a linear effect of Ht on the miltefosine quantification was nevertheless visible in these experiments and therefore a wider range of Ht values was prepared to investigate the relation between Ht and the bias in miltefosine quantification. Human WB was adjusted to five different Ht levels (10, 21, 30, 40 and 51%), spiked at two concentration levels (QCL and QCH) and spotted with a volume of 20 µL. Samples were analyzed in triplicate.

Figure *3* depicts the bias caused by Ht (%, on y-axis) in area ratio of quality control samples prepared in WB with different Ht values relative to standard quality control samples prepared in WB Ht30. The linear trend in the percent bias of the miltefosine concentrations with increasing Ht values relative to Ht30 could be described by equation 1 (R^2=0.9761):

Equation 1: $$Bias_{Ht} = (0.013 \times Ht - 0.359) \times 100\%$$

The same Ht range was spotted at 10, 30, 40 and 50 µL and the linear regression had approximately the same slope regardless of the blood spot volume [data not shown].

12. Clinical evaluation: DBS vs plasma concentrations in patient samples

A total of 16 paired DBS and plasma samples were available from miltefosine-treated Ethiopian HIV co-infected VL patients. Samples originated from the last treatment day, during which miltefosine plasma concentrations exceed the ULOQ. Miltefosine concentrations ranged from 8,420-29,300 and 6,920-29,300 ng/mL for DBS and plasma, respectively. The median of the ratio of the observed miltefosine DBS to plasma concentration is 0.99 (range: 0.83-1.22). The correlation between paired individual observed miltefosine plasma and DBS concentrations is depicted in Figure 4 using a weighted Deming regression. The slope of the weighted regression line was 0.87 [95%CI: 0.70-1.04] with an intercept of 2091 [95%CI: -1132-5313] (Pearson's r = 0.946). The line of true identity, with a regression slope of 1, lies within the 95% CI of the Deming regression line (see Figure 4). This would indicate an approximately equal distribution of miltefosine over blood plasma and erythrocytes.

Miltefosine (indicated as "MIL") plasma concentrations can thus be derived from the observed DBS concentration using the derived Deming regression equation, as follows:

Equation 2: $$[MIL]_{plasma,\ derived} = \frac{([MIL]_{DBS} - 2091)}{0.87}$$

All derived miltefosine plasma concentrations calculated from the observed DBS concentrations using equation 2, were within ±20% of the observed plasma concentration as shown in the Bland-Altman plot (Figure 5).

Large between-patient variability in baseline Ht values is expected in VL patients and Ht typically increases over time during the treatment period when patients recover from their infection. Given the effect of Ht on the miltefosine quantification in DBS established in the bioanalytical validation, the appropriateness of Ht-correction of the clinical DBS concentrations was assessed using the patients' paired DBS and plasma samples. Individual patient Ht values were available for all paired samples, ranging between 23.4% and 44.0% with a median of 30.5%. We tested Ht-correction of the observed DBS concentration for these clinical samples by using equation 1, describing the effect of Ht on the miltefosine quantification in the bioanalytical validation, resulting in equation 3:

Equation 3: $$[\mathrm{MIL}]_{\mathrm{DBS,\ corrected}} = \frac{[\mathrm{MIL}]_{\mathrm{DBS,\ observed}}}{0.641 + 0.013 \times Ht}$$

The correlation between the Ht-corrected DBS concentration and the corresponding observed plasma concentration using a weighted linear Deming regression resulted in a slope of 0.83 [95%CI: 0.73-0.94] with an intercept of 2051 [95%CI: 238-3863] (Pearson's r = 0.951; graph not shown). The 95% CI of both the slope and intercept of the Ht-corrected and non-Ht-corrected regression lines were overlapping, indicating that Ht-correction does not provide a significantly better fit. While all derived plasma concentrations were within 20% of the observed plasma concentration without Ht-correction, two out of the 16 paired samples were outside the ±20% bias relative to the observed plasma concentration when first correcting the DBS concentration for the $\mathrm{Bias_{HT}}$ (see Figure 6). Furthermore, there was no obvious or systematic trend visible in the bias of the derived plasma concentration (no Ht-correction) versus the Ht level (Figure *7*). Based on the clinical validation, correction of miltefosine DBS concentrations for their Ht value appeared not to be appropriate.

DISCUSSION

The here described assay is the first assay to measure miltefosine concentrations in patients using the less invasive DBS sample collection, to facilitate and support future clinical trials investigating new anti-leishmanial treatment regimens encompassing the drug miltefosine. The assay was successfully validated according to FDA/EMA guidelines and EBF recommendations. With this method, miltefosine can be accurately and precisely quantified with an LLOQ of 10 ng/mL, and concentrations as high as 40,000 ng/mL can be analyzed by a 100-fold dilution. Paired miltefosine DBS and plasma samples were collected from 16 HIV co-infected VL patients in Ethiopia. This clinical evaluation demonstrated a good correlation between observed plasma and DBS concentrations. Miltefosine plasma concentrations derived from the observed DBS concentrations using a weighted Deming regression were within 20% of the observed plasma concentration, over a wide range of concentrations. We showed here that the observed miltefosine DBS concentrations are approximately equal to the paired observed plasma concentrations. This indicates an equal distribution of miltefosine between

erythrocytes and plasma in blood of miltefosine-treated VL patients, an observation which has not been shown previously, to the best of our knowledge. DBS samples were found to be stable for at least 162 days up to 37°C, using a simple storage procedure with desiccant packages, which enables storage of the miltefosine PK DBS samples at room temperature in tropical regions.

Influence of Ht on miltefosine DBS measurements

The patients included in our study showed variable Ht-values as described previously (15), with a median of 30.5% which is around the standardized Ht of 30% used for the preparation of calibration standards and QCs in this assay. Despite a linear correlation between Ht and the miltefosine DBS quantification bias observed during the laboratory bioanalytical validation, no such trend in bias due to Ht was found in the clinical application with individual patients' Ht ranging from 23.4% to 44.0%. Neither did Ht-correction significantly improve the calculation of the derived miltefosine plasma concentration from the observed DBS concentration in patient samples. Additionally, four out of 16 patients exceeded the validated Ht range (35.2%, 37.2%, 38.4%, 44.0%), but also for these the observed plasma concentrations were accurately described by the observed DBS concentration, without the need for Ht correction.

These findings show that the observed Ht effect on miltefosine quantification in the bioanalytical validation cannot be confirmed in the clinical validation. Several factors can be hypothesized to have an effect on miltefosine quantification in clinical practice, which could altogether potentially counteract the observed effect of Ht on miltefosine determination. The most general explanation for the Ht effect on analyte quantification is that the Ht impacts the distribution of the applied blood over the filter paper (12). Blood with a high Ht will spread less and therefore the fixed-diameter sub-punch will contain a larger volume of blood than for blood with a lower Ht. It could be debated that when spotting the bioanalytical validation samples with a pipette, more pressure is applied than with a finger-prick spotting in which the drop merely falls onto the paper. This difference in blood flow upon application of the blood spot on the filter paper might theoretically reduce the total blood volume contained in the 3.0 mm punch from the dried blood spot.

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It could also be hypothesized that the blood spot volume is larger for patients with a lower Ht value, due to lower viscosity of the blood leading to a higher blood flow. However, when comparing the blood spot diameter as an indication of blood spot volume (23) for the blood spots in this clinical validation, no such trend was found between Ht and blood spot diameter for the patient samples (R^2=0.002) [data not shown]. Therefore this is not likely to explain the absence of Ht-related bias in the miltefosine quantification of clinical samples.

Additionally, the DBS samples used in the bioanalytical validation differ from the clinical DBS samples in terms of matrix. While the clinical samples are derived from capillary blood obtained by finger puncture, venous blood obtained by venipuncture was used for bioanalytical validation purposes for practical reasons. It has previously been described that the analyte concentrations in these two matrices could differ, mostly explained by the slower distribution equilibrium towards the capillaries (24). However, miltefosine accumulates during treatment and reaches steady-state levels during the last week of treatment in most patients. As the clinical DBS samples are collected one day after the last dose of miltefosine, we do not expect the miltefosine concentration to differ between these two matrices.

Finally, during the bioanalytical validation the effect of Ht is tested while other blood constituents, such as plasma proteins and other blood cells, were kept constant. However, in clinical samples these blood constituents may be variable and potentially also correlated with the Ht value, affecting the miltefosine quantification. For instance, serum albumin levels are significantly lower during active VL infection compared to healthy controls (25), as are Ht levels, and both anemia and low albumin levels were found to be a risk factor for poor clinical outcome in VL (26). A low Ht and low albumin levels are therefore expected to be correlated. Miltefosine is highly protein bound (96-98%) and the majority of the protein-bound fraction (97%) is bound to albumin (27). This could imply that reduced serum albumin levels theoretically lead to an increase in the unbound miltefosine fraction in plasma and correspondingly to an increased distribution of miltefosine towards the erythrocytes (5). The effect of blood protein changes on the quantification of miltefosine, concurrently with low Ht levels, cannot be accounted for in the bioanalytical validation.

In conclusion, various clinical factors potentially affect the miltefosine quantification, cancelling out the systematic bias caused by Ht and making individual Ht correction redundant in clinical practice. Nonetheless, the absence of a bias due to Ht in the clinical samples makes the application of DBS sample collection easier in the field without the explicit need for concurrent Ht measurements and thus allows for DBS sample collection without expensive laboratory equipment.

Applicability of miltefosine DBS sampling method

For the clinical validation we only had a limited number of paired samples available. While there is no strict consensus about the required number of paired samples for method comparisons, the evaluation of forty samples has been proposed (28). However, the collection of additional paired samples in the highly anemic HIV co-infected patients of this study was unfortunately not feasible due to practical limitations and ethical constraints. Paired patient samples were available over a wide but relatively high (>ULOQ) range of miltefosine plasma concentrations between 6,920-29,300 ng/mL. However, as no trend could be observed concerning the effect of Ht on miltefosine quantification from DBS in clinical practice over this wide concentration range, we do not expect that Ht-correction will be needed for lower concentration ranges.

We have demonstrated that DBS sample collection is a valid alternative to plasma sampling for the quantification of miltefosine, which has many practical advantages. DBS sampling is minimally invasive and only requires a minute volume of blood. This is particularly beneficial for the method's application in a pediatric population, a large proportion of VL patients is below 12 years old, but also e.g. in highly anemic HIV co-infected VL patients. Additionally, the DBS collection constitutes a low biohazard risk, by reducing the risk of needle stick incidents when sampling HIV co-infected VL patients. Finally, expensive and logistically challenging cold-chain storage and transport is not required for the DBS samples, simplifying the conduct of PK studies in remote areas where leishmaniasis is endemic and only limited clinical and laboratory infrastructure is available.

TRANSPARENCY DECLARATIONS

None to declare.

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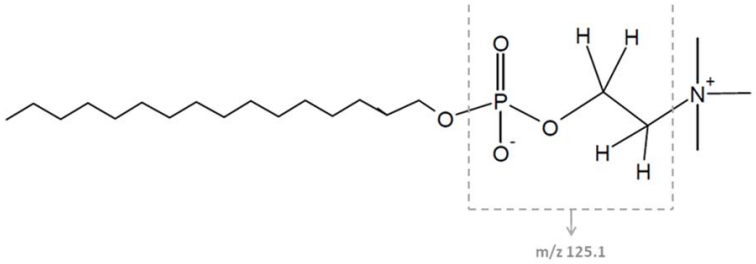
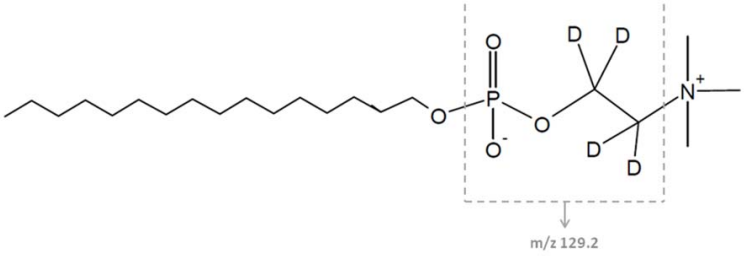
FIGURES AND TABLES



(I) Miltefosine



(II) Miltefosine-D4

Figure 1: Structural formulas of miltefosine and the internal standard miltefosine-D4, indicating the m/z fragments

Parameter		
Run duration	5.0 min	
Ionspray voltage	+4.5 kV	
Turbo gas temperature	400 °C	
Turbo gas flow	7 L/min	
Nebuliser gas	11 psi	
Curtain g as	9 psi	
Collision gas	6 psi	
	Miltefosine	**Miltefosine-D4**
Parent mass	408.5 *m/z*	412.6 *m/z*
Product mass	125.1 *m/z*	129.2 *m/z*
Dwell time	400 ms	400 ms
Collision energy	43 V	43 V
Collision exit potential	22 V	22 V
Declustering potential	71V	71 V
Focussing potential	290 V	290 V
Entrance potential	12V	12 V
Typical retention time	2.6 min	2.6 min

575 Table 1: MS operating parameters for the determination of miltefosine in dried blood spots
576

Run	Nominal conc. (ng/ml)	Bias (%)	CV (%)	# replicates
1	10.1	-9.0	9.1	5
2	10.1	8.1	7.1	5
3	10.1	4.8	19.1	5
Inter-assay	10.1	1.3	14.3	15
1	24.2	1.2	5.8	5
2	24.2	6.3	4.6	5
3	24.2	1.1	6.5	5
Inter-assay	24.2	2.8	5.8	15
1	302	2.0	1.3	5
2	302	-2.5	3.6	5
3	302	11.2	6.2	5
Inter-assay	302	3.6	7.0	15
1	1610	-0.5	5.4	5
2	1610	0.9	5.2	5
3	1610	5.5	3.5	5
Inter-assay	1610	1.9	5.1	15

577 Table 2: Intra-assay and inter-assay accuracy (bias %) and precision (CV%) determined by analyzing quality control samples
578 at four concentrations: LLOQ (10.1 ng/mL), QCL (24.2 ng/mL), QCM (302 ng/mL) and QCH (1610 ng/mL).

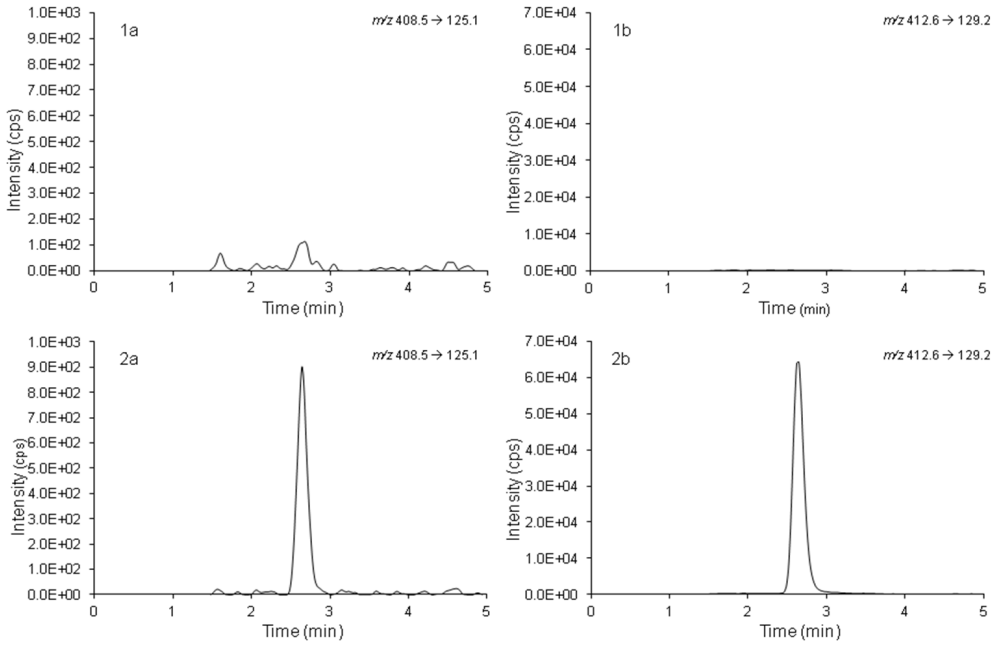
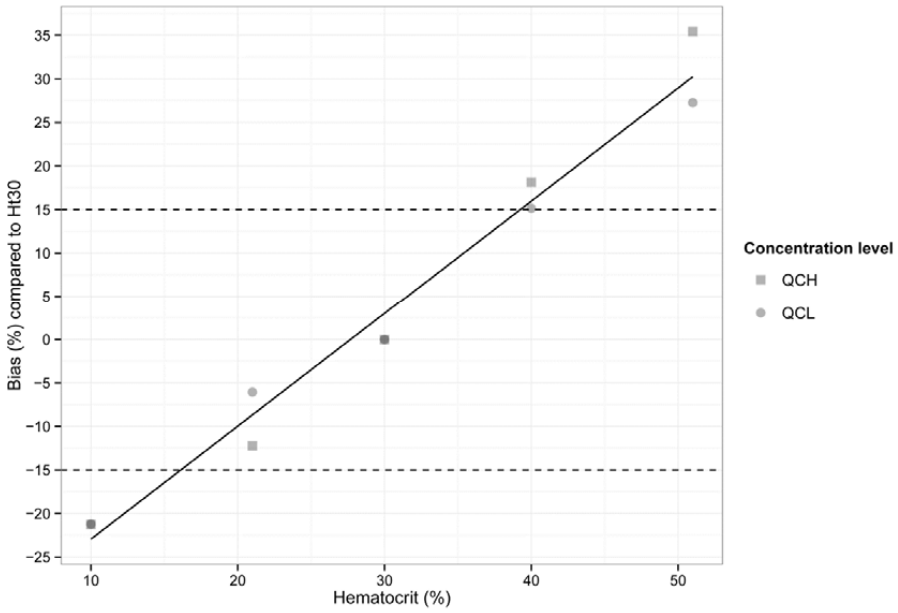


Figure 2: Representative LC-MS/MS ion chromatograms of miltefosine (1a) and internal standard miltefosine-D4(1b) quantified in a double blank DBS and of miltefosine (2a) and miltefosine-D4 (2b) in an LLOQ sample (10.1 ng/mL)



582
583

584 Figure 3: Effect of hematocrit on accuracy of miltefosine quantification at two concentration levels (QCL: 24 ng/mL, QCH:
585 1600 ng/mL) depicted as bias (%) in area ratio compared to Ht30 WB (used for calibration standards). Linear regression line
586 described as BiasHt = (0.013 × Ht -0.359) × 100%. Dotted lines indicate the 15% bias.

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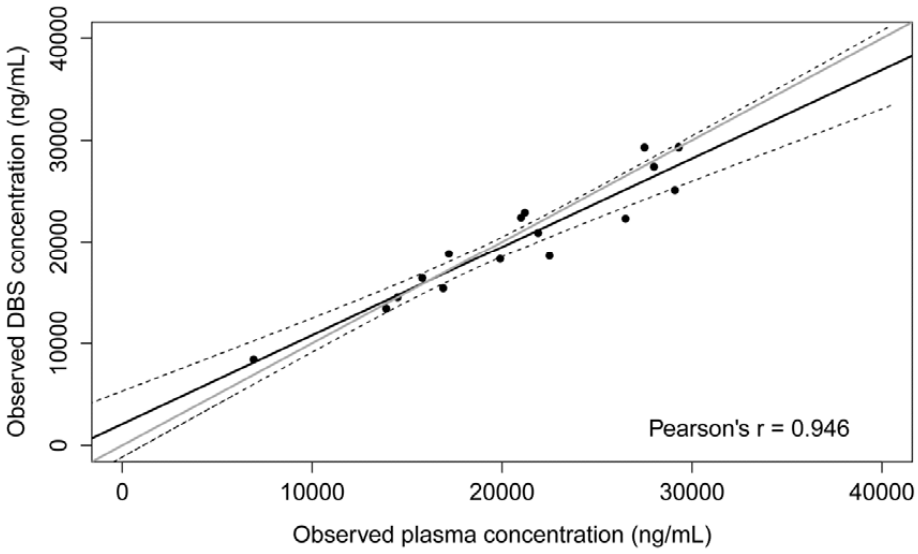
588
589



590

591 *Figure 4: Observed miltefosine dried blood spot concentrations plotted against their corresponding observed plasma*
592 *concentration in paired patient samples (n=16). The weighted Deming fit (2091 + 0.87x, Pearson's r = 0.946) is indicated*
593 *with a solid black line and the dashed black lines show the 95% confidence interval of the fit. The solid grey line indicates the*
594 *line of true identity.*
595

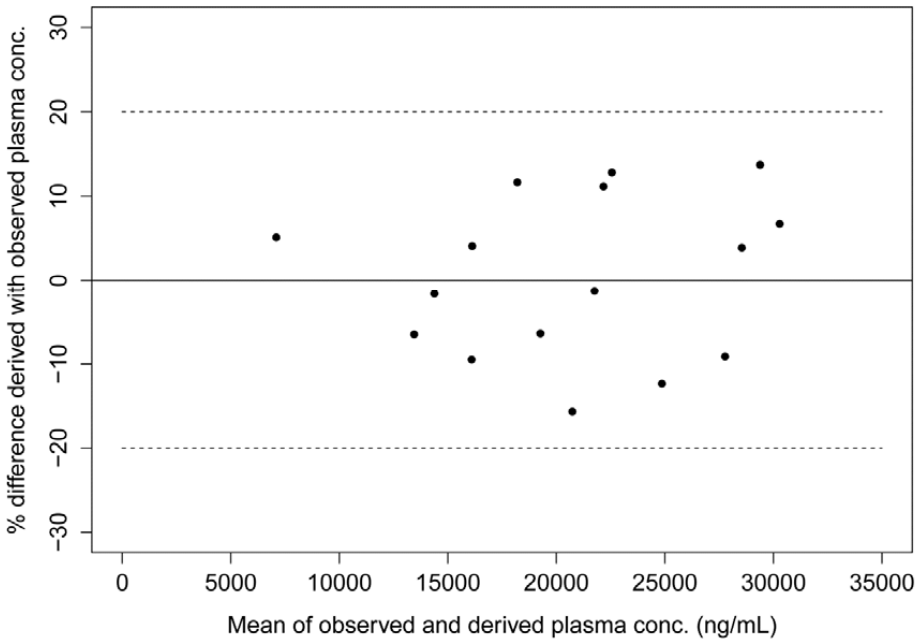


Figure 5: Bland-Altman difference plot depicting the difference between the derived plasma concentration using the Deming regression equation based on the observed DBS concentration, and the observed plasma concentration. The dashed lines represent the 20% bias compared to the observed plasma concentration.

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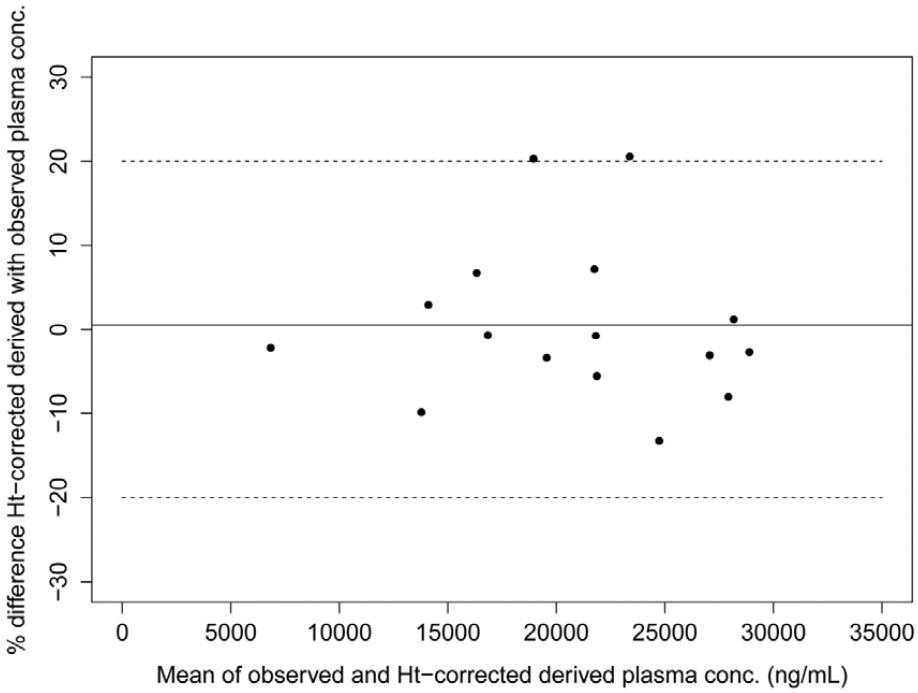

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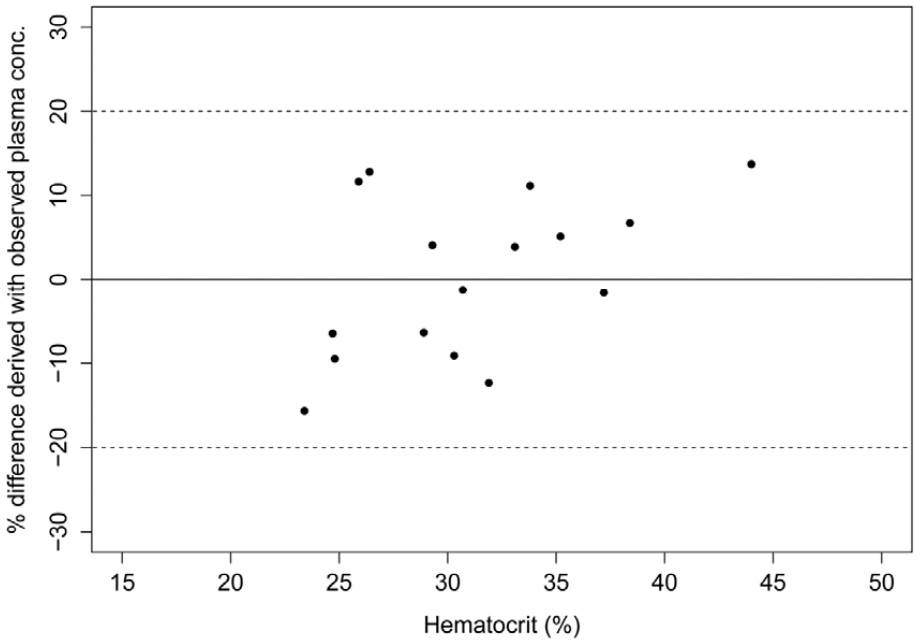
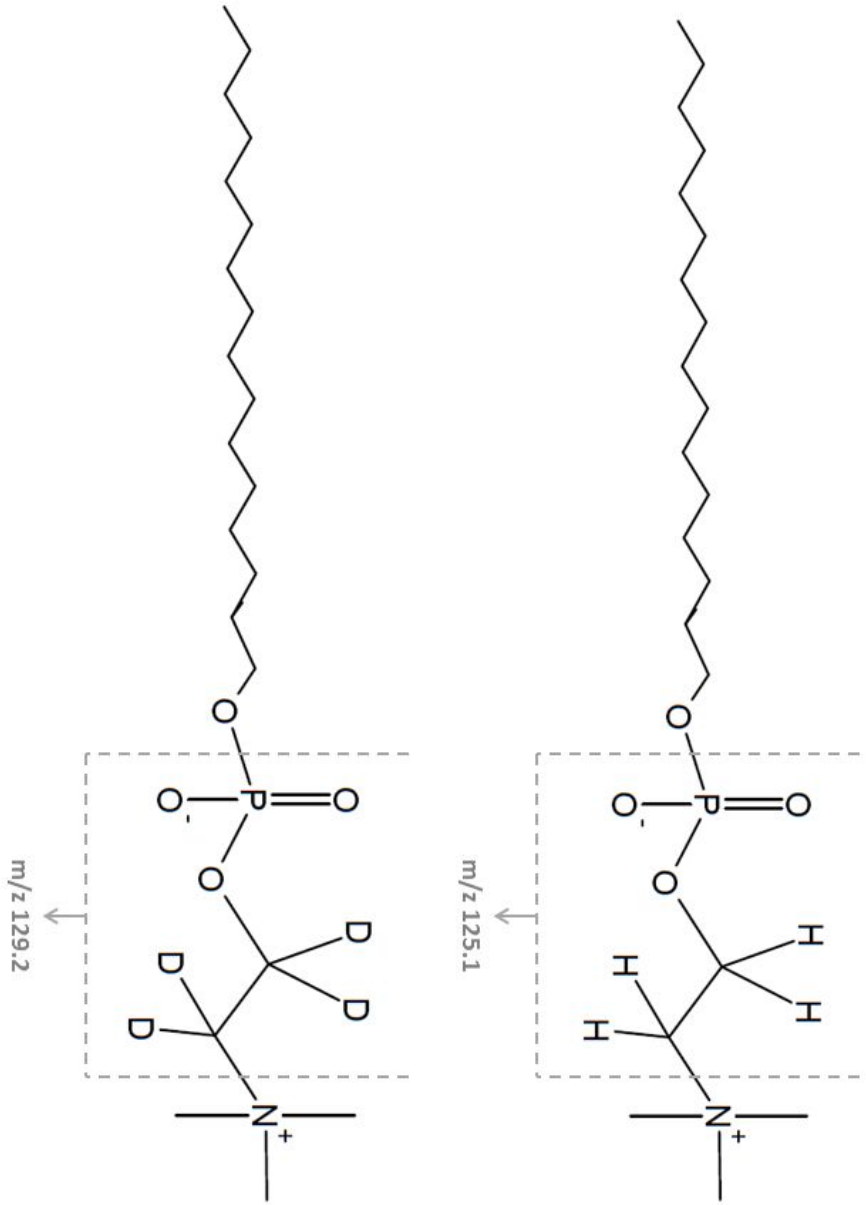
605

606 *Figure 6: Bland-Altman difference plot depicting the difference between the Ht-corrected derived plasma concentration*
607 *using the Deming regression equation based on the Ht-corrected DBS concentration, and the observed plasma*
608 *concentration. The bold line is the mean of the observed difference. The dashed lines represent the 20% bias compared to the*
609 *observed plasma concentration .*



610

611 Figure 7: Difference between the derived plasma concentration using the Deming regression equation based on the observed
612 DBS concentration (without Ht-correction), and the observed plasma concentration versus the hematocrit value. The dashed
613 lines represent the 20% bias compared to observed plasma concentration.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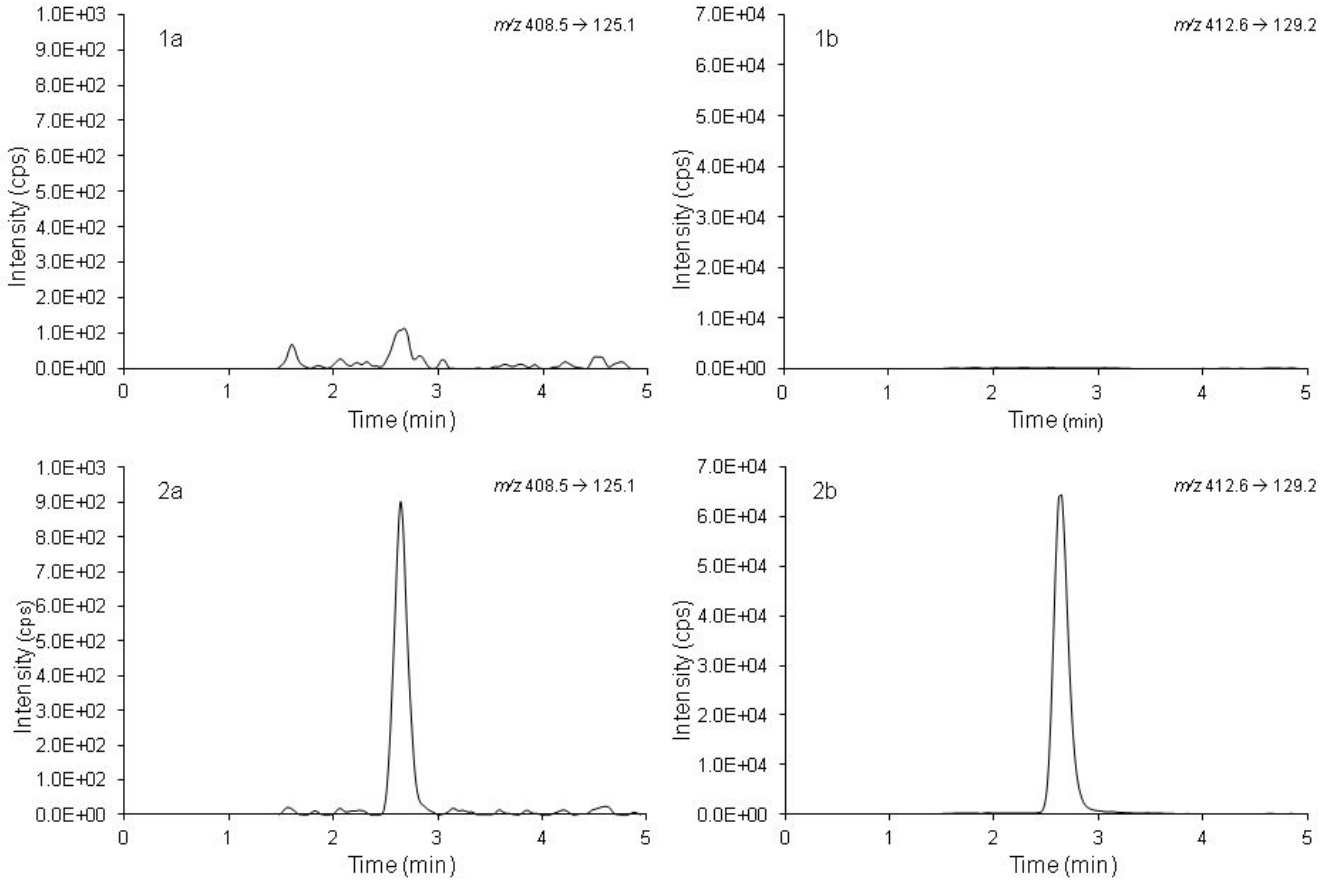
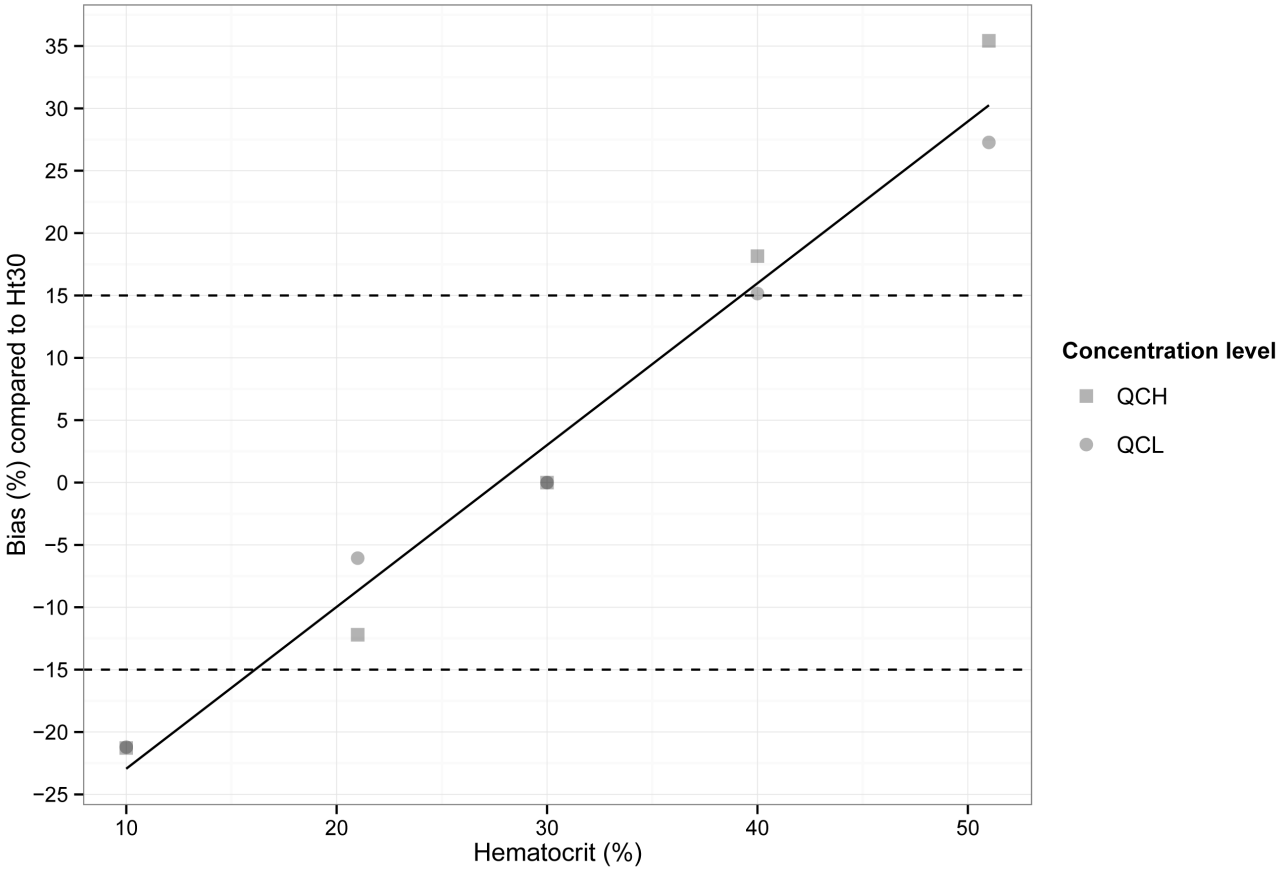
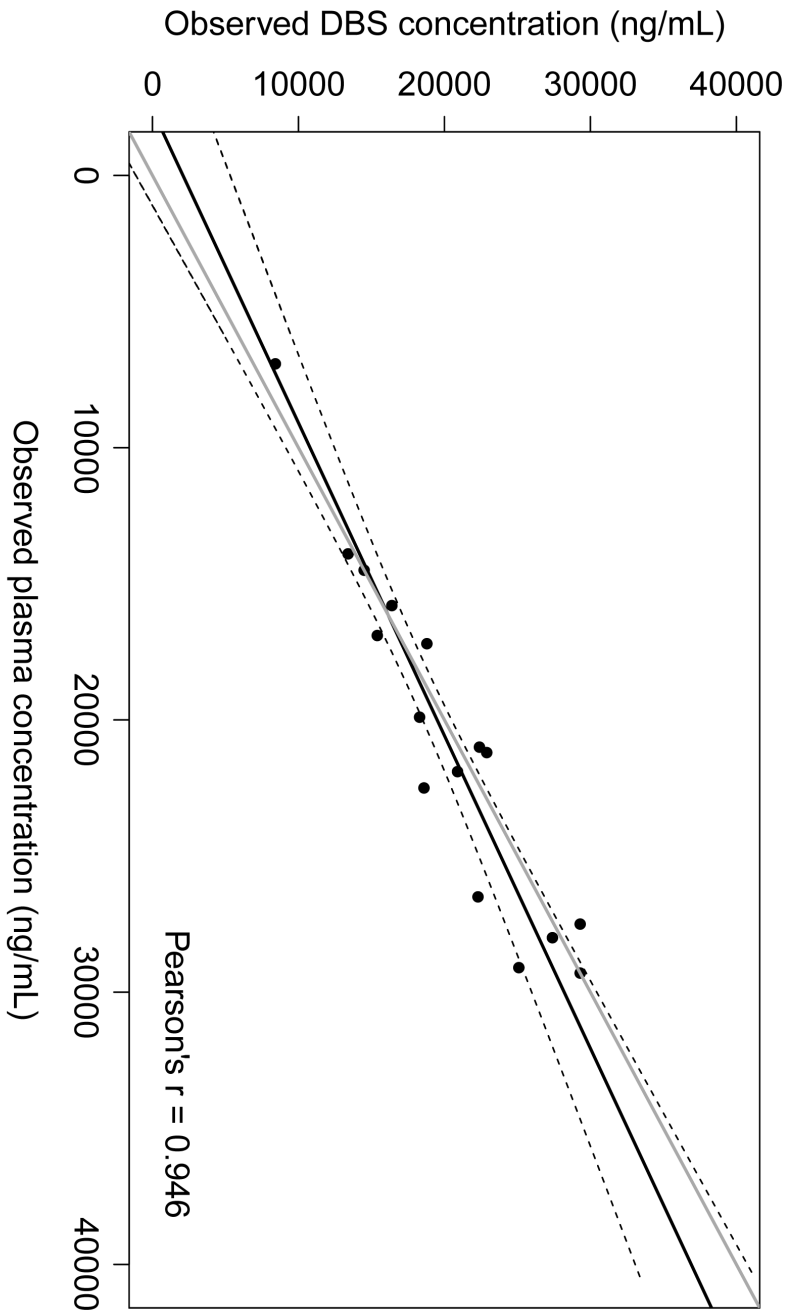
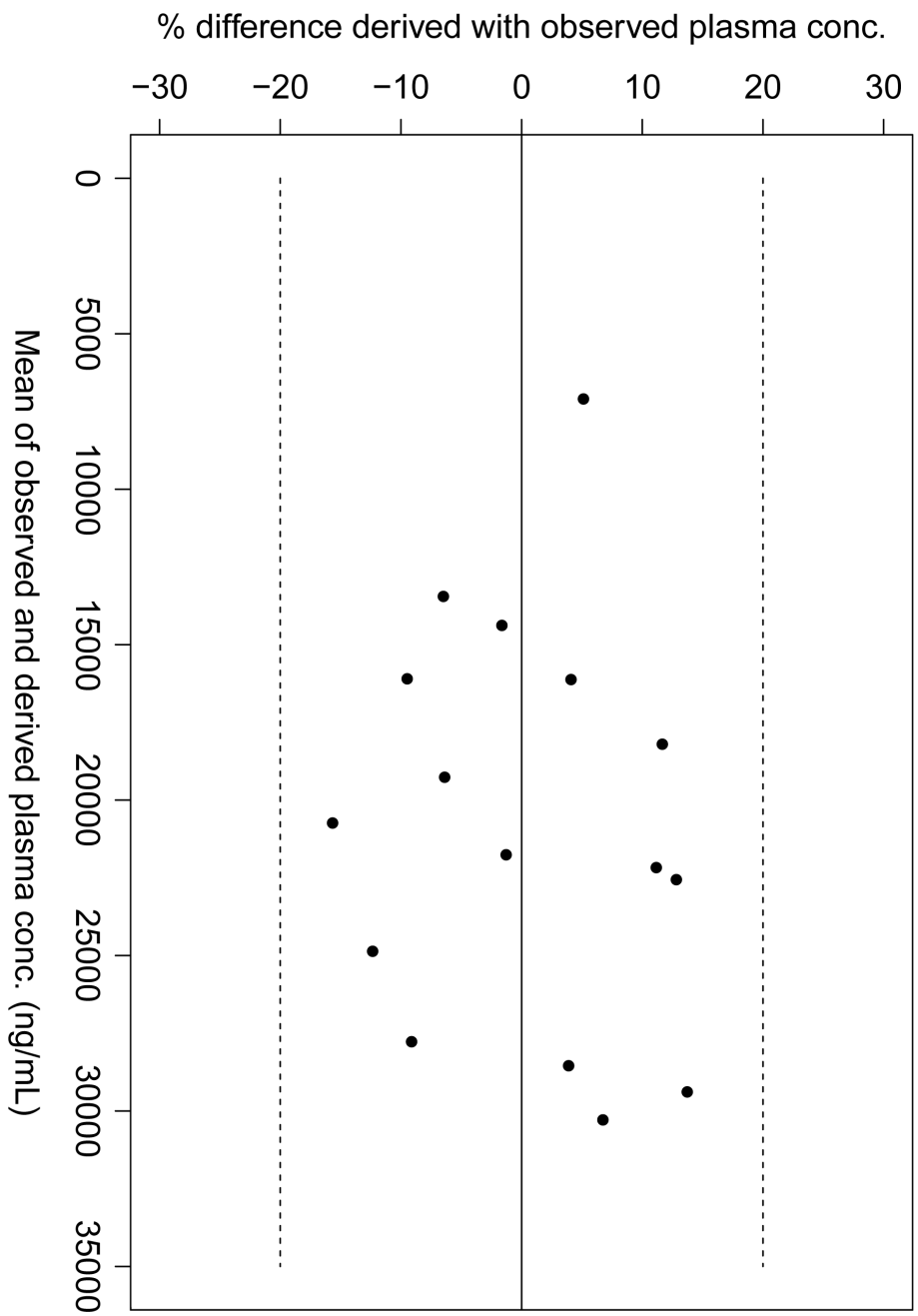
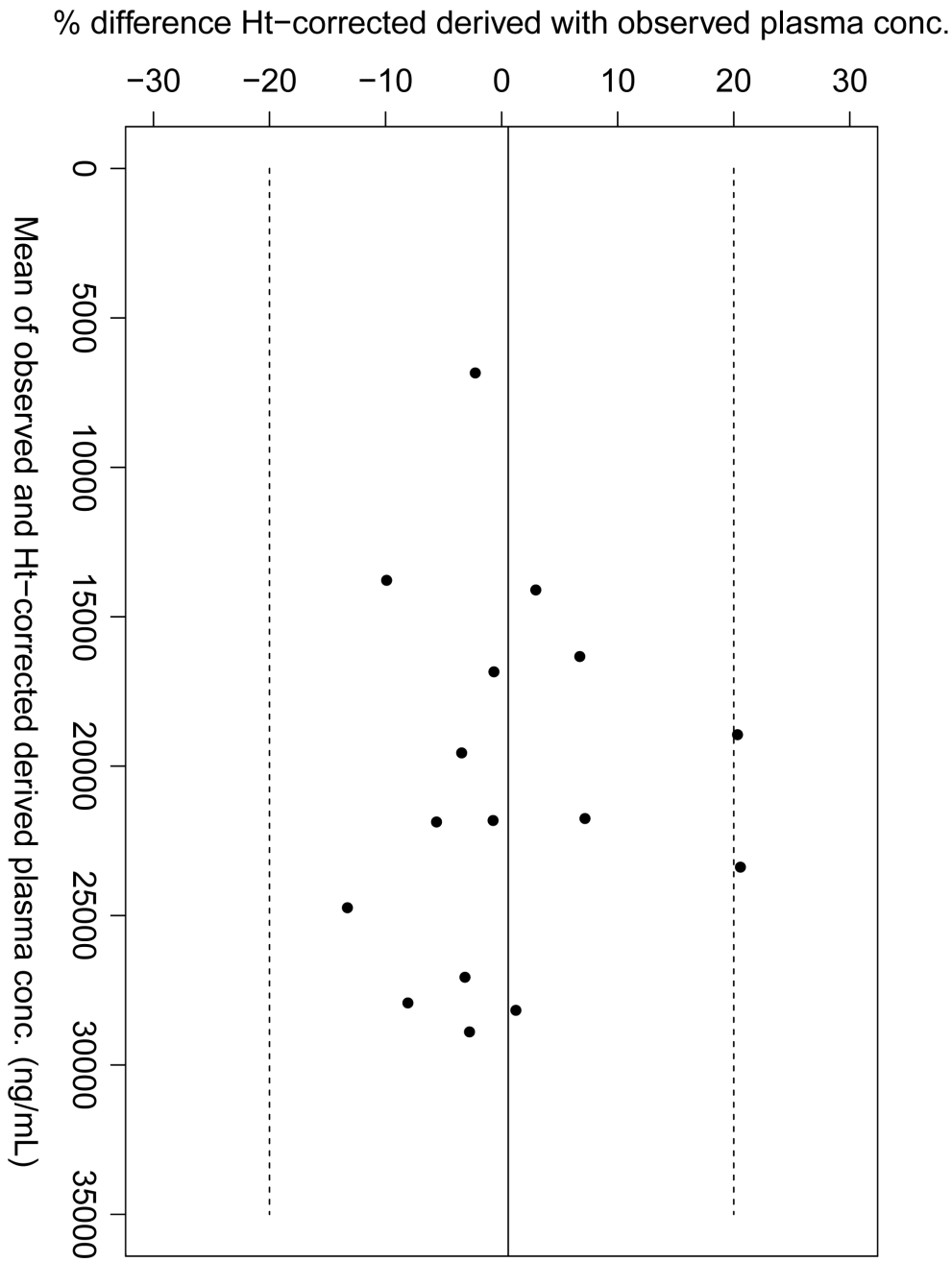
m/z 125.1

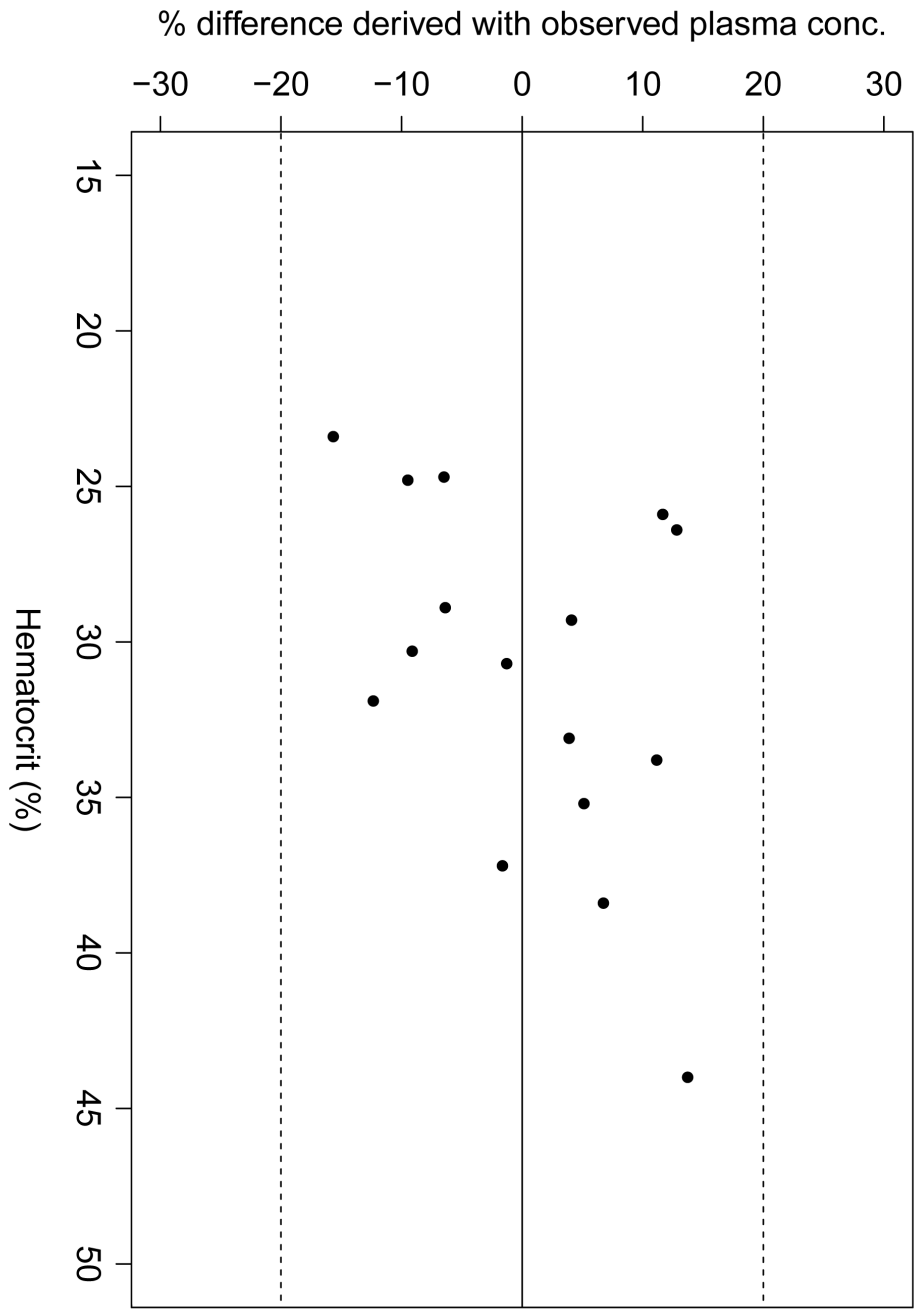





Observed DBS concentration (ng/mL)
0
10000
20000
30000
40000
Observed plasma concentration (ng/mL)
0
10000
20000
30000
40000
Pearson's r = 0.946


% difference derived with observed plasma conc.
−30
−20
−10
0
10
20
30
Mean of observed and derived plasma conc. (ng/mL)
0
5000
10000
15000
20000
25000
30000
35000


% difference Ht−corrected derived with observed plasma conc.
−30
−20
−10
0
10
20
30
Mean of observed and Ht−corrected derived plasma conc. (ng/mL)
0
5000
10000
15000
20000
25000
30000
35000


% difference derived with observed plasma conc.
−30
−20
−10
0
10
20
30
Hematocrit (%)
15
20
25
30
35
40
45
50