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Short communication

Volumetric absorptive microsampling (VAMS) as an alternative to conventional dried blood spots in the quantification of miltefosine in dried blood samples

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ABSTRACT

Miltefosine is an oral agent against the neglected tropical disease leishmaniasis, which is mostly endemic in resource-poor areas. Dried blood spot (DBS) sampling is an attractive alternative to plasma sampling for pharmacokinetic studies in these remote areas, but introduces additional variability in analyte quantification due to possible blood spot inhomogeneity and variability in blood spot volume and haematocrit values. Volumetric absorptive microsampling (VAMS) potentially overcomes a few of these issues as the VAMS device absorbs a fixed volume that is processed as a whole. We developed and validated an LC–MS/MS method for the quantification of miltefosine with this novel sampling technique with good performance in terms of linearity, selectivity, accuracy (bias within ±10.8%), precision (CV% ≤ 11.9%), recovery, carry-over and matrix effect. VAMS samples were stable for at least one month at room temperature and 37 °C. The impact of haematocrit on assay accuracy was reduced compared to conventional DBS sampling, but indicated a declining recovery with increased haematocrit due to haematocrit dependency in recovery from the sampling device. A clinical validation will be required to investigate whether VAMS is an appropriate and cost-effective alternative sampling method to conventional DBS sampling.

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1. Introduction

Miltefosine is currently the only oral drug in the treatment of the neglected tropical diseases cutaneous and visceral leishmaniasis (VL). The drug is now mainly being evaluated in combination therapies in special patient populations such as HIV co-infected VL patients. Since miltefosine exposure is a significant determinant of treatment outcome [1], pharmacokinetic analyses are important in the evaluation of new treatment regimens that include miltefosine.

Our group has recently published a novel bioanalytical assay for the quantification of miltefosine in dried blood spots (DBS) collected on Whatman 903 filter paper [2]. Despite the many advantages of DBS sample collection, this sampling method introduces new variables affecting the accuracy in the quantification of analytes [3–5] such as blood spot volume, blood spot homogeneity and individual time-varying haematocrit (Hct) levels. The most discussed hurdle in the application of this conventional DBS sampling, where a sub-punch of the blood spot is extracted from a DBS card, is the Hct effect on the viscosity of the blood leading to variability in the blood volume collected in the sub-punch taken from the sample for analysis [3]. Also in the bioanalytical validation of the aforementioned method quantifying miltefosine in DBS, there was a Hct effect on the accuracy of the method ranging between −21.3% to +35.4% in an Hct range from 10 to 51% [2]. Hct is a particularly important factor in VL patients, since their Hct levels are typically severely decreased during active disease (median 25%) and increase towards a median of 33% at the end of treatment [6]. Hct issues could be avoided in conventional DBS sampling by whole spot analysis of a volumetrically controlled blood spot applied by a capillary or pipette. However, this adds further complexity to the sampling method which is especially troublesome in

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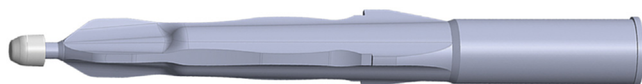

Fig. 1. Picture of the VAMS device Mitra™ (www.neoteryx.com). The VAMS sampler consists of a hydrophilic polymeric tip attached to a plastic handle. The tip absorbs a fixed volume of approximately 10 μL.

the resource-poor endemic VL settings and/or would still require venous sampling.

A new sampling method in which a fixed volume of whole blood can be collected is volumetric absorptive microsampling (VAMS). The VAMS device (depicted in Fig. 1) absorbs a small fixed volume of 10 μL whole blood by wicking onto a porous, hydrophilic tip [7]. At present, a total of six bioanalytical method validations applying VAMS have been published [8–13]. Across a 20–70% Hct range, the variation (CV%) in the median absorbed whole blood volume was only 3.6% [7], demonstrating that the VAMS device accurately and reproducibly absorbs a fixed volume of blood independent of Hct. Inter-laboratory variability in blood volume sampled by six different laboratories was only 8.7% [14]. Compared to venous sampling, the VAMS technique offers the same advantages as conventional DBS sampling: reduced blood sampling volumes, simplification of the sample collection (not requiring cannulae), simplification of pre-treatment methods, reduced costs of shipment and storage at room temperature. In addition, the VAMS device overcomes important issues related to conventional DBS sampling. A fixed volume is absorbed and processed as a whole, which means there is no additional variability of blood spot volume and blood spot homogeneity affecting the accuracy of the method. Moreover, VAMS sampling should in theory overcome the conventional DBS issue of variable blood volumes in sub-punches depending on the Hct level, since the volume collected with the VAMS device should be the same independent of Hct.

In this study, we describe the development and validation of an LC–MS/MS method for the quantification of miltefosine in dried blood collected with the VAMS device. An additional aim of this study was to evaluate the Hct effect on miltefosine quantification in comparison with the previously published conventional DBS method.

2. Methods

2.1. Materials & chemicals

VAMS devices (brand name Mitra™) were purchased from Neoteryx, LLC (Torrance, CA, USA) (Phenomenex, Utrecht, the Netherlands). Miltefosine (Fig. 2) was supplied by Sigma-Aldrich (Zwijndrecht, the Netherlands) and deuterated miltefosine (miltefosine-D4, Fig. 2) was purchased from Alsachim (Illkirch Graffenstaden, France). Methanol (HPLC grade) and water (HPLC grade) were purchased from Biosolve Ltd. (Valkenswaard, the Netherlands). Ammonia (25%) was purchased from Merck (Amsterdam, the Netherlands).

Whole blood was collected from healthy volunteers in K_2EDTA BD Vacutainers® and adjusted to mimic typical Hct values of VL patients (between 23 and 37% [6]) by addition of blood plasma of the same volunteer obtained after whole blood centrifugation. Hct levels were determined with either the Cell Dyn Hematology analyser (Abbot Diagnostics, Lake Forest, IL, USA) or the XN-3000 Hematology analyser (Sysmex, Kobe, Japan). Whole blood was stored at 2–8 °C no longer than one week before the preparation of validation samples.

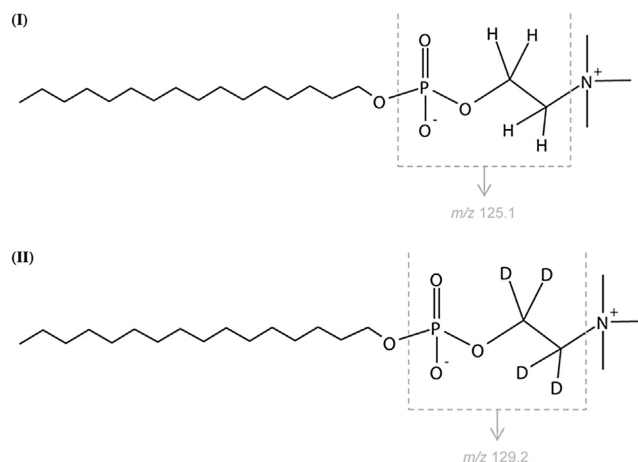

Fig. 2. Chemical structures of (I) miltefosine and (II) miltefosine-D4.

2.2. LC–MS/MS system

Chromatographic separation and MS/MS analysis were performed with the same equipment and under the same settings as described previously in the validation of the conventional DBS method for the quantification of miltefosine [2]. Chromatographic separation was performed using a Gemini C18 analytical column on an HPLC system (Agilent 1100 series; Agilent, Palo Alto, CA, USA). The API-3000 triple-quadrupole mass spectrometer (MS) was used as a detector. LC–MS/MS details can be found in the supplementary information.

2.3. Preparation of calibration standards and quality control samples

Two stock solutions of 1 mg/mL miltefosine in methanol-water (1:1, v/v) were prepared from independent weightings. Separate stocks were used for the preparation of calibration standards and quality control (QC) samples. Working solutions were prepared in methanol-water (1:1, v/v). Calibration standards were subsequently prepared from these working solutions by a 1:20 (v/v) dilution in Hct-adjusted whole blood (Hct value of around 30%) to final nominal concentrations of 10, 20, 200, 750, 1500, 2250, 4000 and 5000 ng/mL. Similarly, QC samples were diluted from working solutions to final concentrations of nominally 10, 25, 450, and 3750 ng/mL (lower limit of quantification [LLOQ], low-level QC [QCL], mid-level QC [QCM], and high-level QC [QCH], respectively). To test the dilution integrity, a sample above the upper limit of quantification (>ULOQ) of 50,000 ng/mL was prepared from a 1:20 (v/v) dilution in Hct-adjusted whole blood (Hct value of around 30%).

Samples were mixed carefully by inversion of the tube and applied to the VAMS device by touching the tip to the blood sample surface. After the tip was completely colored, the contact with the blood surface was extended for two seconds to ensure full absorption, as described previously [7]. Misuse of the VAMS device, such as double-dipping and immersing the tip past the shoulder, was attentively avoided [7]. The device was dried at ambient room temperature for at least three hours.

A stock solution of 1 mg/mL miltefosine-D4 was prepared in methanol-water (1:1, v/v) and diluted to an internal standard (IS) working solution of 400 ng/mL miltefosine-D4 in methanol (IS400).

2.4. Final pre-treatment method

The tip was separated from the holding device in a 2 mL Eppendorf tube to which 175 μL methanol was added. Samples were mixed for 15 min at 1250 rpm. After vortex mixing the samples for an additional 30 s, 100 μL of extract was transferred to a clean 1.5 mL Eppendorf tube. For samples expected to be above the validated range, 10 μL of extract was transferred to a 1.5 mL Eppendorf tube containing 190 μL of methanol and subsequently vortex mixed for 30 s after which 100 μL was transferred to a clean Eppendorf tube. To all samples, 10 μL IS400 was added and samples were vortex mixed for 30 s. The final extract was transferred to vials and injected onto the column.

2.5. Validation

The method validation was based on the FDA and EMA guidelines for bioanalytical method validation [15,16]. The following validation tests were performed: calibration model, accuracy and precision, selectivity, dilution integrity, matrix factor, recovery, carry-over and stability. Additionally, the effect of Hct on analyte quantification was assessed as recommended by the European Bioanalysis Forum (EBF) for DBS methods [17].

2.5.1. Linearity

Fresh calibration standards were prepared in duplicate for all validation runs and injected at the beginning and end of each run. Analyte/IS peak area ratios were plotted against the corresponding nominal concentration and the linear regression was evaluated with both a 1/x and $1/x^2$ weighting factor, where x is the analyte concentration. Back-calculated concentrations should be within ±15% of the nominal concentration (or ±20% for the LLOQ). At least one calibration sample at LLOQ and ULOQ level should be within the requirements in each validation run.

2.5.2. Selectivity

Selectivity was evaluated by collecting whole blood from six different healthy volunteers and preparing a double blank and LLOQ sample for each batch. The chromatograms were evaluated for potential endogenous interferences. Selectivity was considered acceptable if observed interferences were ≤20% of the LLOQ for miltefosine and ≤5% of the IS signal for miltefosine-D4. According to the acceptance criteria, the accuracy and precision of the LLOQ samples should be within ±20% and ≤20%, respectively.

2.5.3. Accuracy, precision and dilution integrity

Intra- and inter-assay precision and accuracy were determined by analysing five processed replicates of the quality control samples (LLOQ, QCL, QCM and QCH) in three separate validation runs. Five replicates of the >ULOQ were prepared, diluted and analysed in one validation run to determine dilution integrity. The accuracy of the method is expressed as the bias from the measured analyte concentration and should be within ±15% of the nominal concentration (±20% for LLOQ). The intra-run bias (%) is calculated as the bias of the mean measured concentration per run, compared to the nominal concentration. The inter-run bias (%) is calculated as the bias of the overall mean measured concentration over all three runs, compared to the nominal concentration. The intra-run precision of the method was determined by calculating the percent coefficient of variation (%CV) for each set of replicates per run and was considered acceptable if ≤15% (≤20% for LLOQ). To calculate the inter-run variation, a one-way ANOVA was used.

2.5.4. Recovery and matrix factor

To evaluate recovery and the matrix factor, blank samples were prepared from six different whole blood batches in duplicate as described in paragraph 2.4. From the same six batches of blood a QCL and QCH sample were prepared in singular. The recovery was calculated per separate whole blood batch from the ratio of the processed QC sample response to the response of the blank extract spiked with the required level of analyte to obtain the same miltefosine final extract concentration as the QC sample. The required analyte amount to be added was determined with the calculated average blood wicking volume as described in the Mitra™ certificate of conformance.

The matrix factor was calculated for each whole blood batch as the ratio of the spiked blank extract response versus the mean of the matrix free neat solution response (n = 3).

2.5.5. Carry-over

The carry-over of the LC–MS/MS was tested by injection of two double blank processed samples directly after the injection of a processed ULOQ sample. The peak areas of the blanks were compared to the mean area of the five LLOQ replicates. Carry-over was considered acceptable if ≤20% of the LLOQ for miltefosine and ≤5% of the IS signal for miltefosine-D4.

2.5.6. Stability

Short-term stability was tested for five days at room temperature (22 ± 3 °C) at the QCL and QCH concentration level. Long-term stability was tested for one month storage at room temperature (22 ± 3 °C) and at 37 ± 3 °C at the QCL and QCH concentration level. The VAMS devices were stored in the supplied clamshell storage containers in the dark (Neoteryx, LLC, Torrance, CA, USA).

Final extract stability was tested up to 5 days at 2–8 °C at the QCL and QCH concentration level. Stock solutions of 1 mg/mL in methanol-water (1:1, v/v) were stored at −20 °C for 28 months. Working solutions were stored at −20 °C for 40 months at miltefosine concentrations of 80 and 40,000 ng/mL in methanol-water (1:1, v/v).

2.5.7. Hct effect on method accuracy

To determine the assay bias over a range of Hct, VAMS samples were prepared from K_2-EDTA whole blood adjusted to 10, 20, 41 and 50% Hct and spiked at QCL (25 ng/mL) and QCH (3750 ng/mL) level in three-fold for each Hct level. Calibration standards were prepared as described above in whole blood adjusted to a Hct value of 30% (Section 2.3). Accuracy (bias%) and precision (CV%) were considered acceptable across the Hct range if within ±15% and ≤15%, respectively.

Conventional DBS samples were prepared as described previously [2] in whole blood adjusted to Hct 14, 25, 38 and 50%. DBS samples were prepared at the QCL (24 ng/mL) and QCH (1610 ng/mL) concentration level in three-fold for each Hct level. Concentrations were quantified using calibration standards spiked with miltefosine to whole blood adjusted to a Hct value of 29%.

For both VAMS and DBS samples the bias was calculated as the difference between the mean calculated concentration and the nominal concentration.

3. Results & discussion

3.1. Linearity

Calibration curves of analyte/IS peak area ratio versus nominal analyte concentration were evaluated and when a weighting factor of $1/x^2$ weighting was applied, the bias across the range was improved considerably. Linear regression correlation coefficients (R^2) were ≥0.9955 in all cases. All back-calculated calibration standard concentrations were within ±15% of their nominal value and coefficients of variation were below 15%. The linear range of

Table 1
Selectivity results for determination of miltefosine in dried blood samples collected with VAMS device prepared in six batches of blank whole blood — accuracy of the LLOQ (nominal concentration 9.99 ng/mL) per batch and relative interference as percentage of LLOQ in double blank per batch.

Batch whole blood	Hct (%)	Measured miltefosine concentration (ng/mL)	Accuracy LLOQ (Bias, %)	Relative interference double blank (% of LLOQ)
#1	37	11.2	12.1	9.9
#2	43	11.4	14.1	5.4
#3	49	9.87	−1.2	7.3
#4	48	9.95	−0.4	4.5
#5	42	11.9	19.1	7.5
#6	48	9.61	−3.8	7.0

10–5000 ng/mL for this method is therefore wider than for the previously published plasma (4–1000 ng/mL [18]) and conventional DBS method (10–2000 ng/mL [2]). Given the wide range of miltefosine concentrations encountered in patients [19], this wider bioanalytical range will reduce the number of samples requiring dilution.

3.2. Selectivity

The results for the selectivity of the validated method are shown in Table 1. There were no unexpected endogenous interferences >20% of LLOQ for miltefosine, nor >5% of the IS signal. The method was therefore considered to be selective. Additionally, the LLOQ could be analyzed precisely and accurately for all six batches (bias within ±19.1%, %CV 9.0%).

3.3. Accuracy, precision and dilution integrity

The intra- and inter-run mean biases, shown in Table 2, were within ±10.8% (within ±7.9% for the LLOQ), and thus meets the criteria for assay validation. Representative chromatograms are depicted in Fig. 3. Intra- and inter-run precision were below 9.9% for all validation runs (≤11.9% for LLOQ), and therefore also meet the established criteria. The intra-run bias and precision for the 20-fold dilution of the <ULOQ sample (50,000 ng/mL) were 0.1% and 4.8%, respectively. The here validated method was therefore considered accurate and precise in a concentration-range between 10 and 50,000 ng/mL.

The expected miltefosine concentration-range in patients covers a wide range from 10 to 50,000 ng/mL [19]. This emphasizes the importance of dilution integrity for this method, as samples above 5000 ng/mL have to be diluted up to 10 times. As dried blood samples cannot be diluted before the sample pre-treatment, samples above the quantitation limit were diluted prior to addition of the internal standard. As described in the pre-treatment method, this required volume transfer of all samples – including calibration standards and QC samples – before the internal standard was added, which could potentially introduce additional variability. This validation revealed however no negative impact of this dilution method on the precision of the method.

3.4. Matrix factor and recovery

The absolute mean matrix factor was 0.637 (range 0.606–0.674) and 0.691 (range 0.532–0.802) at QCL and QCH, respectively. The mean normalized matrix factor (matrix factor analyte/matrix factor IS) was 0.992 (range 0.941–1.033) and 0.996 (range 0.963–1.017) at QCL and QCH, respectively, indicating that the deuterated IS miltefosine-D4 effectively compensates for matrix effects. Mean normalized sample pre-treatment recovery was 69.7% (range 60.5–79.2%) and 70.6% (range 66.9–78.4%) as QCL and QCH, respectively. The matrix factor and recovery were reproducible for the six different batches of whole blood (%CV ≤10.8). Hct values of the six different batches ranged between 30 and 42%.

During the development phase of the pre-treatment method, extraction recovery with 5% $ZnSO_4 \cdot 7H_2O$ in water, as advised by the VAMS manufacturer for small molecule extraction, was compared to extraction with methanol using VAMS samples prepared at 1600 ng/mL miltefosine in whole blood of 33 and 48% Hct. The variation in extraction recovery between Hct levels was lower for methanol (68.7% versus 60.3%, for 33 and 48% Hct respectively) than for extraction with 5% $ZnSO_4 \cdot 7H_2O$ in water (103% versus 78.5%). Furthermore the extraction with methanol resulted in an approximately three times higher absolute MS response compared to 5% $ZnSO_4 \cdot 7H_2O$ in water, due to the ion suppressive effects of the sulphate ions in the latter.

3.5. Carry-over

The response in the blank samples after injection of the ULOQ was ≤19.8% (0% for the IS) of the mean response at the LLOQ. Therefore, the carry-over is considered acceptable.

Table 2
Accuracy and precision for the determination of miltefosine in dried blood samples collected with the VAMS device.

QC level	Spiked miltefosine concentration (ng/mL)	Run	Intra-run mean bias (%)	Intra-run precision (%CV)	Inter-run mean bias (%)	Inter-run precision (%CV)
LLOQ	10	1	4.2	11.9	4.4	–[a]
		2	7.9	8.9		
		3	1.2	5.6		
QCL	25	1	2.2	9.9	−0.4	3.9
		2	1.7	4.8		
		3	−5.2	4.9		
QCM	450	1	10.8	7.5	4.5	4.8
		2	0.1	7.4		
		3	2.7	4.9		
QCH	3750	1	3.4	5.5	2.4	–[a]
		2	−0.4	6.4		
		3	4.3	2.3		
>ULOQ	50,000	1	0.1	4.8		

[a] Inter-run precision could not be calculated (mean square between the groups is less than the mean square within the groups), meaning that there is no additional significant variation in performing the assay in different runs.

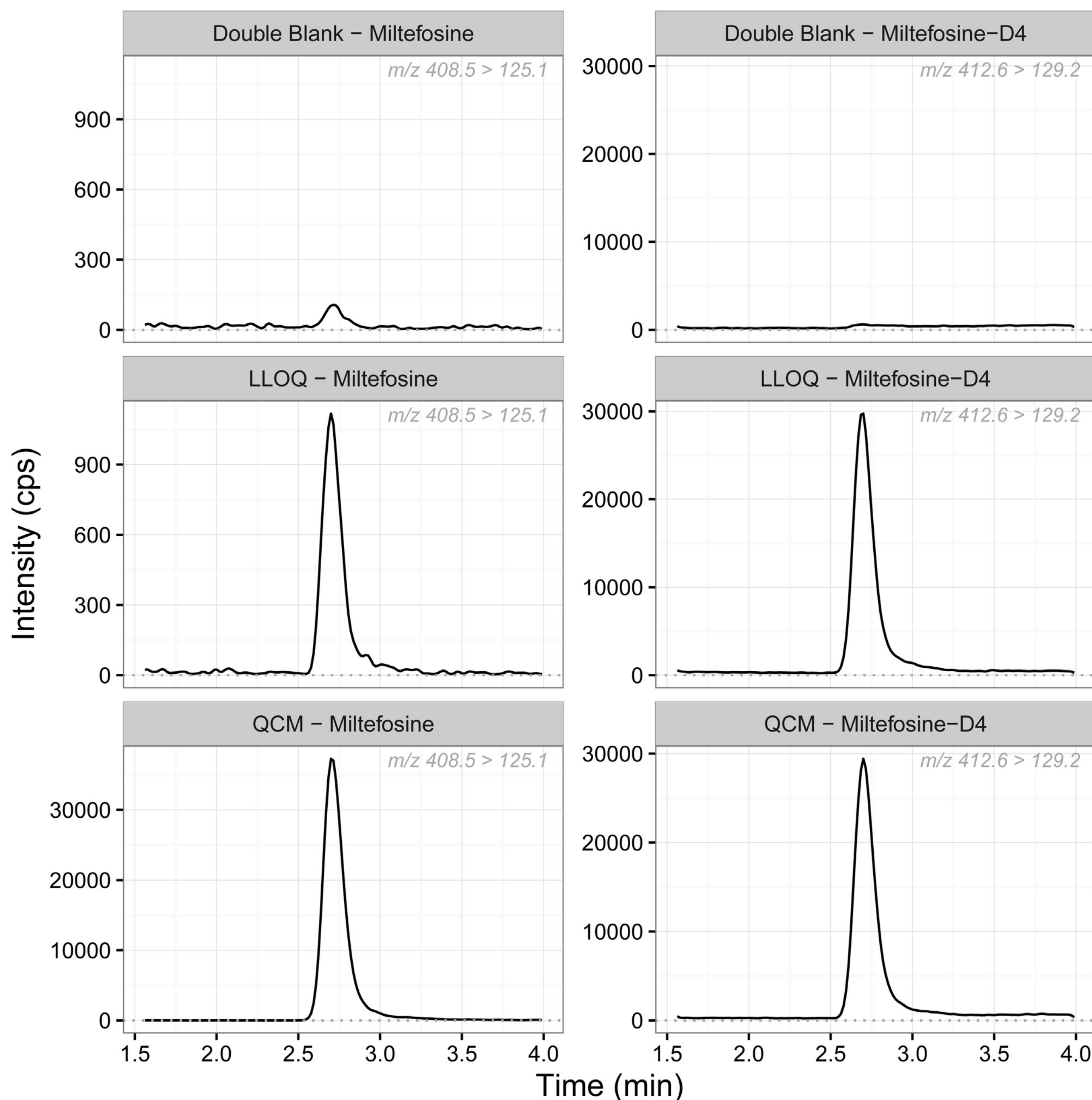

Fig. 3. LC–MS/MS chromatograms obtained from double blank, LLOQ (10 ng/mL) and QCM (450 ng/mL) samples. Left is miltefosine, right is the deuterated internal standard miltefosine-D4.

3.6. Stability

Miltefosine was stable in dried VAMS samples for at least one month at QCL and QCH levels at a room temperature of 22 ± 3 °C (bias −8.0 and −6.6%, respectively) and at 37 °C (bias −9.7 and −13.0%, respectively). Since a declining trend in accuracy of the analysed miltefosine concentrations was observed, it could be considered to store samples in the freezer for longer storage periods (stability under these conditions was not evaluated).

Final extract stability was acceptable for at least 5 days at 2–8 °C (bias −2.0 and −3.6% for QCL and QCH, respectively). At −20 °C, stock solutions (1 mg/mL) were stable for at least 28 months with a bias of 1.9%. Working solutions were stable for at least 40 months at −20 °C (bias within ±4.4%).

3.7. Hematocrit effect on accuracy

As previously observed during the bioanalytical validation of the conventional DBS method [2], there was a trend visible in the impact of Hct on miltefosine quantification bias for conventional DBS (Fig. 4). The bias in the measured miltefosine concentration was positively correlated with the Hct value of the whole blood: at low Hct values (14%) the bias was around −20% and at high Hct values (50%) the bias was around +25% (Fig. 4), compared to whole blood calibration standards with an Hct value of 29%. This is in accordance with the published validation, in which the miltefosine DBS method could only be validated between 20 and 35% (biases within ±15%) [2]. In addition to the effect of Hct, the accuracy of the method was influenced by variability in blood spot volume and inhomogeneity of the spot [2].

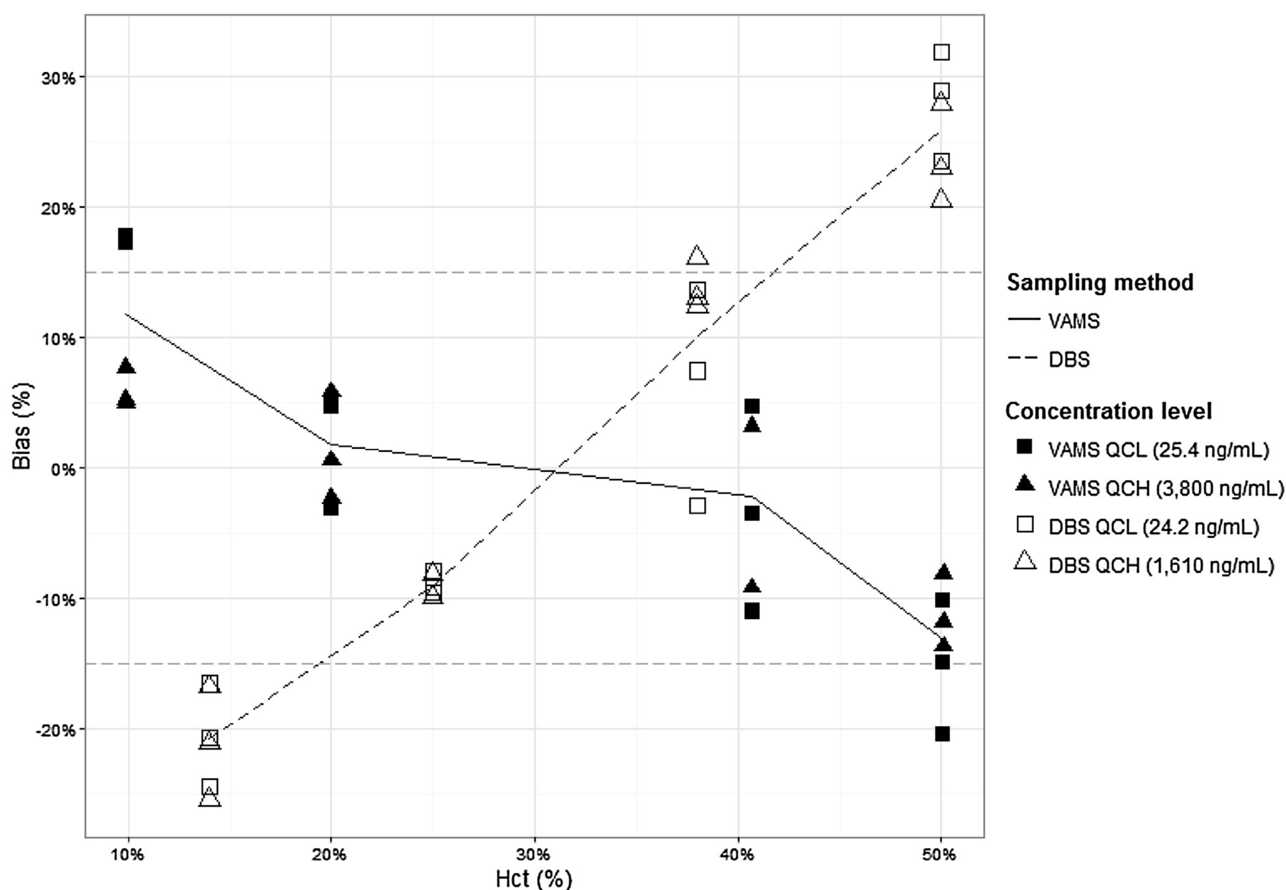

Fig. 4. Hct effect on accuracies of the VAMS sampling method versus conventional DBS (on Whatman 903 filter paper) sampling, depicted as bias of calculated compared to nominal miltefosine concentration. Dotted line depicts the DBS sampling method, solid line depicts the VAMS sampling method. Open symbols depict the DBS sampling method, closed symbols depict the VAMS sampling method. Squared symbols depict the QCL concentration level, triangle symbols depict the QCH concentration level (though at different concentrations between sampling methods).

The Hct effect on accuracy for the VAMS sampling methodology is also depicted in Fig. 4. For both concentration levels (QCL and QCH) the accuracy (depicted as bias%), was inversely correlated with the Hct value with a positive bias for low Hct levels and a negative bias at higher Hct levels. While the bias was less pronounced than for conventional DBS sampling (in a range from Hct 10–50%, only the Hct 10% QCL values were outside the acceptable ±15% bias), an opposite trend was observed for this sampling method.

The Hct impact on accuracy was most probably attributable to the effect of Hct on the analyte recovery described in paragraph 3.4 (at 1600 ng/mL, the recovery was 69% for Hct 33% and 60% for Hct 48%). A total of six method validations have been described using the VAMS sample collection method for analyte quantification in whole blood [8–13], where four of these tested the effect of Hct on analyte recovery. All four studies found a lower recovery for higher Hct levels [8,11–13], which has been explained by the larger amount of erythrocytes entrapping the analyte in the pores of the VAMS device tip, obstructing analyte extraction [12]. The higher positive bias in lower Hct levels was also observed in our study.

In this VAMS method, accuracy and precision were acceptable in an extended Hct range of Hct 20–50%, compared to Hct 20–35% for conventional DBS sampling. The acceptable 15% bias in quantification was only just exceeded for the QCL level at Hct 10%, which even in the case of VL is an extreme that is rarely observed in clinical practice. Recently it was found that adding a sonication step to the sample pre-treatment could possibly improve the Hct-dependent recovery bias [13]. If a wider range of Hct values is expected in clinical practice than 20–50%, the addition of a sonication step in the pre-treatment method could be evaluated for its effect on recovery from the VAMS device.

3.8. Practical considerations

The advantage of the VAMS sampling method over other controlled volumetric methods – such as the use of capillaries to apply blood to DBS cards – is its ease of use in the clinic. This is of special importance in the remote VL endemic areas without appropriate laboratory set-up.

The trend towards a decrease in stability within one month storage at room temperature and 37 °C is an issue of concern. There were no long-term stability issues with miltefosine in conventional DBS up to five months [2]. Long-term stability of the dried VAMS sample (>7 days) was only assessed in three studies, of which two encountered problems with long-term stability [8,10], but one did not [12]. For the applicability of this method in clinical practice in VL endemic areas, longer stability times are required and these should therefore be evaluated before application in the clinic.

A clinical evaluation of this method is scheduled to evaluate the VAMS sampling method as an appropriate alternative for conventional DBS sampling. While a correlation of the Hct value on miltefosine quantification could be observed in the bioanalytical validation of the conventional DBS method, no obvious or systematic effect of Hct could be observed in its clinical validation. This indicates that many other factors (also including blood spot volume and blood spot homogeneity in the case of DBS sampling) influence

miltefosine quantification in DBS in clinical practice. Additionally, while this new sampling method is easy to use in a clinical setting, the method is more prone to misuse such as double-dipping or insufficient absorption. Clinical evaluation of the VAMS method will thus be required to investigate whether the variability in miltefosine quantification in dried blood is improved in clinical practice in comparison to DBS sampling.

One disadvantage of the VAMS sampling method is the relatively high costs in comparison to DBS Whatman-903 cards. As a Whatman-903 protein saver card fits five samples, one VAMS sample is approximately five times more expensive than a conventional DBS sample. Especially in resource-poor settings, this is an important consideration for the applicability of this sampling method and cost-effectiveness should be evaluated.

4. Conclusion

The novel and simple volumetric absorptive micro-sampling (VAMS) device, was evaluated for its applicability in quantitative miltefosine bioanalysis. Though the preparation of calibration standards and quality control samples is more time-consuming than for conventional DBS, the pre-treatment method is fast and simple with reproducible recovery. The analytical method showed a good performance in terms of linearity ($R^2 > 0.9955$), accuracy (bias within $\pm 10.8\%$) and precision ($CV\% \leq 11.9\%$).

In this bioanalytical validation, the VAMS collection process effectively diminished the effect of Hct on miltefosine quantification compared to conventional DBS sampling, however, a reversed Hct bias was introduced with this sampling method. In addition to the decreased Hct impact, this method eliminated additional variabilities due to blood spot volume and blood spot homogeneity. In conclusion, the bioanalytical method validation with the VAMS sampling technique showed a better performance compared to conventional DBS sampling.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2016.12.012.

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