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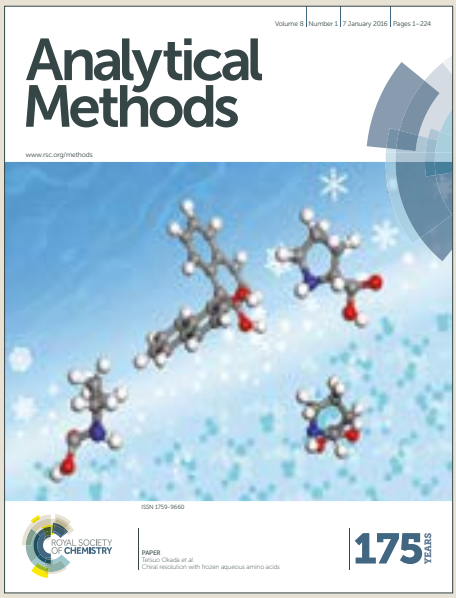

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Development and validation of a LC-MS/MS method for ivermectin quantification in dried blood spots: Application to a pharmacokinetic study in *Trichuris trichiura*-infected adults

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Abstract

Ivermectin serves as a good addition to the small group of medications for soil-transmitted helminths, particularly *Trichuris trichiura* infections. So far, ivermectin has been poorly characterized in clinical trials to treat *T. trichiura;* especially information on its pharmacokinetic (PK) behavior in infected pediatric populations is missing. Existing approaches to quantify ivermectin in human matrices are time-consuming, limited to matrixes of high volume, which excludes the more ethical micro-blood sampling and mostly do not distinguish between ivermectin and its metabolites. Thus, a sensitive, selective and rapid liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed to quantify ivermectin extracted from dried blood spots (DBS), plasma and blood. Method validation was performed on accuracy, precision, sensitivity, selectivity, linearity and stability. While the quantification of ivermectin in plasma and DBS were successfully validated, blood samples failed validation due to insufficient stability and robustness. The method was applied to samples from a clinical study with 11 adult volunteers from rural Côte d'Ivoire infected with *T. trichiura* sampled at 11 time points. Good agreement of the time-concentration profiles and PK parameters of plasma and DBS samples were achieved with *e.g.*, a maximal concentration of 51.6 and 40.1 ng/mL, respectively and identical time to reach maximal concentration of 3.9 h. Comparison of the results by Bland-Altman analysis resulted in high consistency. The less invasive and more patient-friendly DBS micro-blood sampling technique will be useful in future clinical trials to evaluate ivermectin's PK profile in larger populations.

1. Introduction

The avermectin family was discovered in the 1970s and soon thereafter, the derivative ivermectin was developed revealing superior activity against many parasitic worms.[1,2] In 1981, ivermectin was approved to the market as a veterinary anthelminthic drug and in 1987 it was first utilized in human medicine to treat *Onchocerca volvulus*. Donation and mass drug administration programs were soon thereafter initiated to deliver ivermectin to Africa and South America to reach onchocerciasis and a few decades later lymphatic filariasis patients.[1,3] In tropical and sub-tropical settings, co-infections are common and hence, additional efficacy was documented against other parasitic infections including the soil-transmitted helminths (STH).[4,5] The number of infected individuals with STHs is estimated at 1.5 billion and the recommended drugs against STH infections (albendazole, mebendazole, pyrantel pamoate and levamisole) have only poor effect on *Trichuris trichiura*.[6,7]

Ivermectin, however, is still, despite its large-scale use rather poorly characterized for human use.[8] A few clinical studies have been performed on *T. trichiura* including ivermectin or ivermectin co-administration to further evaluate its efficacy against STH infections.[9,10] However, further evidence is required to fully understand the pharmacokinetic (PK) and –dynamic profile of ivermectin in correlation to different doses and safety. Even though some PK studies of ivermectin have been performed, all of them included a low number of exclusively adult volunteers who were healthy or infected with *Onchocerca volvulus*.[11–18] Venous blood sampling was performed in these studies to obtain plasma samples, the only matrix that has been used for ivermectin PK analysis so far. Given the broad interest of the use of ivermectin as treatment for *T. trichiura*, scabies as well as an agent to reduce malaria transmission,[19,20] additional PK studies are required, including children, which are the primary focus for the treatment of STH infections. However, when children are participating in a clinical trial, venous blood sampling is challenging and unethical. Micro-blood sampling (*e.g.*, the dried blood spot (DBS) technique) offers an appropriate alternative for PK studies in this vulnerable population. It is less invasive, less painful, simpler and more rapidly to perform. Additionally, DBS do not require cooling or freezing and are less likely to cause an infection risk for nurses and researchers.[21–23] However, a micro-blood sampling technique has not yet been established for the quantification of ivermectin.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the gold standard for drug quantification. Nevertheless, most analytic methods to quantify ivermectin have been established by high-performance liquid chromatography (HPLC), where the drug was reacted to a fluorescent counterpart.[14,24–26] Even though ivermectin is only poorly metabolized, the established HPLC methods do not distinguish between ivermectin and its metabolites as all compounds are derivatized. Only Kitzman *et al.* developed a HPLC method, which is sufficiently long to separate and quantify ivermectin only.[13] However, a single run of 32 minutes is not applicable when a large number of samples is analyzed. The analysis of ivermectin in plasma, milk or other matrices by mass spectrometry has been described but the methods cannot be applied to micro-blood samples because large sample volumes are required.[27–30]

For the first time, an analytical LC-MS/MS method was developed and validated for the quantification of ivermectin from DBS samples. For comparison, the analytical method was also established for plasma and blood matrices. A PK study with 11 adult volunteers from rural Côte d'Ivoire infected with *T. trichiura* was performed. Participants received orally the standard dose of ivermectin (200 µg/mL) and both DBS and venous samples were withdrawn

at 11 time points post-treatment. Ivermectin was extracted and quantified to illustrate the robustness of the DBS sampling method and the comparability to plasma concentrations.

2. Materials and Methods

2.1 Chemicals and material

Ivermectin (powder, 96% B1A), formic acid (LC-MS grade) and ammonium acetate (LC-MS grade) were purchased from Sigma-Aldrich (Buchs, Switzerland). Ivermectin-d_2 was synthesized by Toronto research chemicals (Ontario, Canada). Ivermectin tablets (3 mg) were kindly provided by ELEA (Buenos Aires, Argentina). Ultrapure water was prepared using a Millipore water purification system (Milli-Q® Advantage A10, Merck, Darmstadt, Germany). LC-MS grade solvents, acetonitrile and isopropanol, and Whatman® protein saver cards 903 were purchased from Merck KGaA (Darmstadt, Germany). SOLAμ solid phase extraction (SPE) plates HPR were obtained from Thermo Fisher Scientific (Reinach, Switzerland). Ivermectin stock solutions were prepared and extraction steps performed in protein low-binding material (Vaudaux-Eppendorf AG, Basel, Switzerland).

2.2 LC-MS/MS instrumentation and settings

Fragment separation and identification was performed on a 1260 Infinity LC System connected to a 6460 triple quad LC-MS (Agilent Technologies, Santa Clara, CA, USA). The autosampler Thermostat (1200 series, Agilent Technologies) was used for sample injection and cooling (4 ºC).

Fragment specific mass spectrometer parameters were optimized by direct infusion of 1 µg/mL ivermectin dissolved in acetonitrile to the mass spectrometer and are summarized in **table 1**. Selected reaction monitoring (SRM) of the positively charged fragment of ivermectin and ivermectin-d_2 was performed for detection (**figure 1**). The general settings of the mass spectrometer were as follows: gas temperature 250 ºC, gas flow 5 L/min, nebulizer 30 psi, sheath gas temperature 300 ºC, sheath gas flow 11 L/min, and capillary voltage 5000 V.

Liquid chromatography was performed on a Luna C8 column (30 x 2.0 mm, 3 µm particle size, 100 Å; Phenomenex, Torrance, CA USA). Ammonium acetate prepared with ultrapure water (mobile phase A, 0.4 mM) and acetonitrile (mobile phase B) served as eluents. Formic acid was added to both eluents (0.1% v/v). A gradient elution was performed (**table 2**) with a flow rate of 0.5 mL/min. The autosampler syringe and injection valve was cleaned three times with isopropanol-H_2O (1:1 v/v) after each sample injection. The software Agilent MassHunter Quantitative Analysis was used for data analysis.

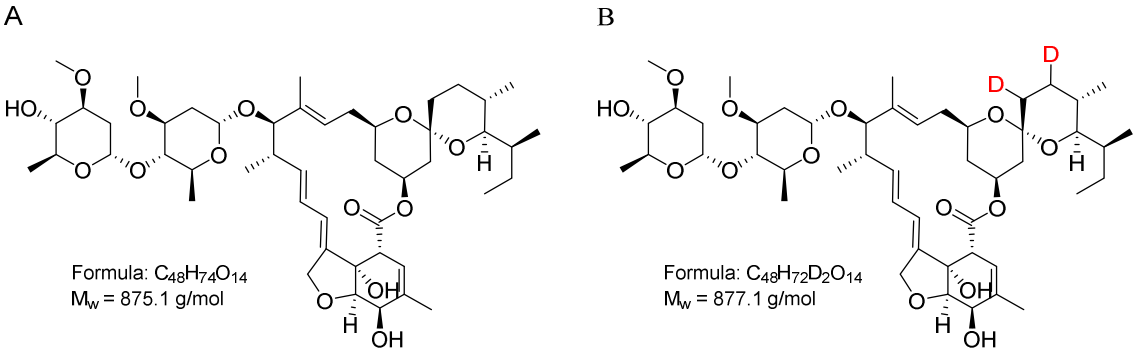

Figure 1. Molecular structure of (A) ivermectin and (B) ivermectin-d_2.

Table 1. Fragment specific mass spectrometry parameters.

Parameter	**Product Ion 307** Ivermectin	**Product ion 571** Ivermectin-d_2
Dwell	100	80
Fragmentor voltage	135	135
Collision Energy	20	8
Cell accelerator voltage	6	2

Table 2. Gradient elution of liquid chromatography.

Time (min)	**Mobile phase B (%)**
0.00	40
0.20	40
0.50	95
2.50	95
2.55	40
4.00	40

2.3 Blood, plasma and dried blood spot (DBS) sample procession

2.3.1 Preparation of calibration and quality control samples

Human blood was obtained from the local blood donation center (Basel, Switzerland) and kept in heparin coated vacutainer tubes to prevent coagulation of blood. The stock solution of ivermectin was prepared in acetonitrile (1 mg/mL) and stored at -20 ºC. Working solutions of ivermectin were obtained by serial dilutions in acetonitrile:H_2O (1:1 v/v). Quality control (QC) and calibration samples were prepared by spiking plasma, blood and DBS with working solutions (50:1 v/v) in the range of 2–200 ng/mL. The final concentration of organic solvent in the spiked plasma and blood samples was < 2%.

According to FDA guidelines, each validation set of samples consisted of at least eight calibration samples (including the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ)), six blank samples (matrix without internal standard) and six zero

samples (matrix with internal standard).[31] Additionally, six QC replicates at the LLOQ, low, medium and high concentration covering the whole calibration range were included in each experiment. QC and calibration samples derived from plasma were always spiked freshly on the day of analysis, blood and DBS samples were prepared and stored at -20 ºC.

QC samples, 2, 3, 100 and 180 ng/mL for plasma analysis and 3, 5, 100 and 180 ng/mL for blood and DBS samples, were included in each run. QC samples were always prepared in six replicates of different human sources.

2.3.2 Sample extraction

The stock solution of ivermectin-d_2 (internal standard, 1 mg/mL) was prepared in acetonitrile and stored at -20 ºC. The extraction solvent 1 (acetonitrile/H_2O (4:1 v/v), ES1) and 2 (acetonitrile/H_2O (5:1 v/v), ES2) were spiked with 0.01 ng of the internal standard. 100 µL plasma or blood samples were mixed with 100 µL or 120 µL of the extraction solvent ES1 or ES2, respectively and agitated for 10 min at 2000 rpm at room temperature (rt).

Two circular spots (ø 8 mm) were punched from each DBS card, 400 µL H_2O was added and agitated at rt for 10 min at 2000 rpm. Then, 400 µL of ES1 was added and again agitated under the same conditions. Thereafter, DBS samples were sonicated in an ultrasonic bath (TPC-280, M. Scherrer AG, Zuzwil, Switzerland) for 15 min at rt.

The samples (plasma, blood and DBS) were centrifuged for 10 min at 3000 x *g*. The supernatant was transferred to a pre-conditioned SPE-96 well plate. According to the manufacturer's protocol, SPE plates were first equilibrated with 200 µL acetonitrile, followed by conditioning with 200 µL H_2O. Once the samples were loaded onto the columns, they were washed with 25% acetonitrile and eluted twice in 25 µL acetonitrile. 10 µL of each sample was injected to the LC-MS/MS system for analysis.

2.3.3 Impact of hematocrit on DBS and blood sample analysis

The effect of hematocrit was evaluated for DBS and blood sample analysis. The hematocrit of QC samples (n = 6 from different human donors) was adjusted with plasma to 20–50% to cover the hematocrit range expected in the study population. Calibration samples were prepared with 35% or 40% hematocrit. The hematocrit values of QC samples were adjusted in all experiments to ensure accuracy and precision of the method.

2.4 Method validation

Method validation for the analysis of ivermectin was performed according to the U.S. FDA guidelines and the following characteristics were validated: stability, selectivity, sensitivity, accuracy, precision, recovery, matrix effect and linearity.[31]

2.4.1 Stability

Different conditions of field and laboratory work were mimicked to test the stability of ivermectin in blood, plasma and DBS. Extracted ivermectin samples were incubated at rt for 4 h and at 4 ºC for 72 h (autosampler stability). One set of spiked samples was stored at -80 ºC for 2 months (long-term stability) and another set was three times frozen for 24 h at -80 ºC followed by thawing to rt (freeze-thaw cycles). Additionally, long-term stability at rt was evaluated for DBS cards (2–3 months). QC samples (low, middle and high) were prepared and kept under the specified conditions. Extracted samples were analyzed and compared to calibration samples. Ivermectin was considered stable when the results did not deviate more than ± 15% from the nominal value of freshly prepared calibrators.

2.4.2 Selectivity and Sensitivity

Blank human plasma and DBS samples were processed from six different human sources to validate the limit of detection (LOD) of each matrix. The signal of LLOQ should be at least 5x of LOD and should not deviate more than ± 20% from the nominal concentration.

2.4.3 Accuracy, Precision

The accuracy and precision of the analytical method was tested with four QC concentrations (LLOQ, low, medium and high) each prepared in six replicates of different blood or plasma sources. Within-run (intra-assay) assessed the precision and accuracy of three validation sets in a single analytical run and between-run (inter-assay) recorded three validation sets on three different days. Results were compared to the nominal value obtained from calibration samples. The results of QC samples were accepted when 4/6 samples were within ± 15% of the nominal value and within ± 20% of the nominal value for the LLOQ. Accuracy is presented as the percentage ratio of measured to the nominal concentration and precision is illustrated as the coefficient of variation (CV [%]).

2.4.4 Recovery and matrix effect

Relative recovery (RRE) and matrix effect (ME) were analyzed with samples of four human sources. QC sample concentrations (low, medium and high) were prepared for analyzing RRE and ME. RRE is obtained by comparing zero blank plasma or DBS that is spiked with ivermectin after extraction to the corresponding extracted samples. ME was determined by comparing extracted zero blank matrix that is spiked after extraction to the corresponding ivermectin concentrations prepared in acetonitrile.

2.4.5 Linearity

Calibration samples were accepted when 75% of the samples were ± 15% (± 20 for LLOQ) of the nominal value. The coefficient of determination (r^2) had to be ≥ 0.99.

2.5 Method application

The validated LC-MS/MS method was applied to samples of a PK clinical trial in rural Côte d'Ivoire with 11 adult volunteers (≥ 18 years of age) infected with *T. trichiura*. Diagnosis was performed by analyzing stool samples with the Kato Katz technique. Participants were orally treated with the standard dose of 200 µg/kg ivermectin. Ethical approval was obtained from the Ethical Committee of Northwestern and Central Switzerland (2017-00250) and the Comité d'Ethique et de la Recherche of the Ministry of Health in Côte d'Ivoire (052/fMSHP/CNER-kp). Informed consent was obtained from all patients. The study was registered at the ISRCTN registry (no. ISRCTN15871729).

Patients received a fatty breakfast (oily fish on bread) since a fatty meal is known to enhance ivermectin's bioavailability.[32] Venous and micro-blood sampling was performed at 0, 1, 2, 4, 6, 7, 8, 9, 24, 48, 72 h post-treatment. Venous blood (~4 mL) was collected in EDTA covered vacutainer tubes (BD, Allschwil, Switzerland) to prevent coagulation. The blood was centrifuged to obtain plasma. Plasma samples were stored at -20 ºC at the clinical trial site. Additionally, microsampling was performed at the same time points by taking capillary blood from the same individuals. Sterile finger-prickers were used to puncture the tip of a finger obtaining a blood drop. Lithium heparin coated capillaries were loaded with blood, which was dropped onto DBS cards (~60 µL per spot). This was performed in four replicates for each patient and time point. The DBS were allowed to dry for at least 2 h and then stored at -20 ºC at the clinical trial site in sealed plastic bags containing silica desiccants. Plasma and DBS samples were shipped to Basel, Switzerland with dried ice and stored at -80 ºC until processed for analysis.

2.6 Data analysis

The following PK parameters were obtained by non-compartmental analysis using WinNonlin (5.2, Certara, Princeton, NJ, USA): maximum ivermectin concentration (C_{max}), time to reach C_{max} (t_{max}), time in which half of the absorbed drug is eliminated (half-life $t_{1/2}$), area under the curve determined until last positive concentration (AUC).

Bland-Altman analysis was performed to evaluate the agreement between plasma *vs* DBS samples. The difference (%) of ivermectin concentration extracted from the matrixes was plotted *vs* the average concentration. Additionally, an unpaired t test was performed following a F test to compare variances of PK parameters. Statistical analysis was performed with Prism 6.01 (GraphPad, CA, USA).

3. Results and Discussion

3.1 Method development

The number of anthelminthic drugs in human medicine is very limited and no candidates are currently in the pipeline. Drug resistance on anthelminthics has been already observed in veterinary medicine,[33–35] a development that must be prevented in humans. Ivermectin with its broad antiparasitic activity is a promising candidate for the treatment of STH infections. However, proper scientific information is missing on efficacy, safety, PK and –pharmacodynamics, particularly for STH infections. Several articles describe the analytical quantification of ivermectin extracted from animal or human plasma or food samples by HPLC mostly detecting a fluorescent counterpart of the drug,[13,25,36] in which ivermectin does not get separated from its metabolites. The PK profiles of the metabolites remain unknown and most likely differ from ivermectin's characteristics.[13,37,38] Additionally, other analytical approaches have been tested such as capillary electrophoresis or gas chromatography.[39,40]

LC-MS/MS is the gold standard for quantifying individual analytes. Two articles describe the analysis of ivermectin by mass spectrometry in plasma, but they are not applicable to micro-blood samples because large plasma volumes are required.[27,28] Ivermectin remains a challenging candidate to be quantified in DBS samples caused by the limited blood volume in combination with its low concentration in the blood after oral delivery.[13,15] This is due to its moderate bioavailability and the low dose of treatment. Additionally, ivermectin poorly ionizes in the mass spectrometer leading to low sensitivity. Based on this information, a careful and stepwise LC-MS/MS method and extraction procedure development was performed.

First, the parent mass of ivermectin was tested in the positive ionization mode. The parent mass ionized with H^+ (*m/z* 875.5 $[M + H]^+$) could not be detected, but the ammonium (*m/z* 892.5 $[M + NH_4]^+$) and sodium counterparts (*m/z* 897.5 $[M + Na]^+$) were reproducibly observed. Sodium ionized molecules fragment poorly in a MS and thus, the ammonium adduct was chosen for analysis.[27] SRM scans were performed with best results for 892.5 → 307 *m/z* and 892.5 → 569 *m/z* fragments. Mass spectrometer source parameters were optimized to increase peak intensity (*e.g.*, gas temperature and flow, nebulizer pressure). Thereafter, fragment specific parameters (**table 1**) were improved for ivermectin and the internal standard. Due to its higher peak intensity, fragment 892.5 → 307 *m/z* was chosen for quantification.

Subsequently, LC conditions were adjusted by testing several columns (C18, C8 and Synergy Polar-RP) with ammonium acetate buffer (mobile phase A, 0.4 mM) and acetonitrile (mobile phase B) both spiked with 0.1% formic acid. First, an isocratic method was performed followed by testing different ratios of mobile phases (20–100% mobile phase B). Highest signal intensity was achieved with a Luna C8 column (30 x 2.0 mm, 3 µm) and a gradient eluent shown in **table 2** with a flow rate of 0.5 mL/min.

In a next step, the extraction method of ivermectin from blood, plasma and DBS was evaluated. Plasma is the standard matrix used for PK studies. Additionally, blood samples were collected to explain possible discrepancy between ivermectin's concentration in DBS and plasma. A classical approach was tested for plasma samples, where proteins were simply precipitated with an organic solvent. Ivermectin recovery was evaluated with acetonitrile, methanol, acetone and ethyl acetate as extraction solvents. Additionally, all solvents were tested in an aqueous mixture. Best results were achieved with a mixture of acetonitrile/H_2O (4:1 v/v, ES1) and a shaking time of 10 min at rt. Longer shaking, heating and the inclusion of sonication were tested with no additional positive effect. Simple filtration did not lead to a highly purified analyte solution and a significant matrix effect was observed. Thus, SPE was performed to maximize signal intensity in the mass spectrometer.

The same extraction method was performed with blood samples. Blood cells and proteins could not be entirely aggregated with the extraction solvent (ES1), which caused unsatisfactory results of the SPE purification. The fraction of organic solvent was increased to 5:1 acetonitrile/H_2O (v/v, ES2), which improved the purification steps and thus overall results.

The punched spot size of DBS was maximized to increase the blood volume analyzed and thus ivermectin amount in the sample. The DBS cards offer a spot size of 12 mm. Blood was spotted with a capillary in a pre-test after pricking a finger to evaluate which blood spot size can be used conveniently at the clinical trial site and an 8 mm spot size was consequently chosen. In order to increase signal intensity in the MS, two spots were used for analysis. Ivermectin extraction from DBS with only acetonitrile or an aqueous mixture of it did not yield satisfying results. Therefore, whole blood was first extracted from DBS with water and thereafter acetonitrile/H_2O (4:1 v/v) was added to extract ivermectin. An additional sonication step further improved MS signals. SPE was performed to yield highly purified samples and to concentrate the samples to lower volume.

3.2 Method validation

Stability of ivermectin in plasma, blood and DBS was determined under different conditions mimicking field and laboratory work (**table 3**). Three QC concentrations (low, middle and high) were extracted and stored at rt for 4 h and at 4 ºC for 72 h. High accuracy was obtained with low deviation (< 11%) for all three matrixes. DBS samples (not extracted) were stored at rt for 2.5 months to evaluate its stability under the conditions at the clinical trial site when no freezer is available. Extracted samples retained high accuracy and precision (**table 3**). Three freeze/thaw cycles were performed with sets of samples, which were subsequently extracted and analyzed. Ivermectin was proven stable in plasma and DBS with high accuracy and precision (CV: 0.9–7%). Spiked blood samples kept under the latter condition yielded ineligible accuracy (81.3–108%, required range: 85–115%) with low precision (CV: ≤ 22%). Additionally, long term stability was tested by storing samples for 2–3 months at -80 ºC. Ivermectin in plasma and DBS samples retained stability, whereas blood samples did not achieve acceptable accuracy and precision (CV > 15%). Thus, ivermectin

samples were found stable under all conditions in plasma and DBS, but storage of blood samples did not yield the required accuracy and precision and thus, failed the validation. Therefore, blood samples were not further evaluated in this work and conclusively, blood is not the matrix of choice when ivermectin is analyzed.

Table 3. Stability of ivermectin in plasma, blood and DBS.

Condition	Matrix	$c_{nominal}$ [ng/mL]	$c_{ø}$ [ng/mL]	Accuracy ± CV [%]
Room temperature (4 h, extracted)	Plasma	3	2.88	96.0 ± 10.9
		100	114	114 ± 1.17
		180	198	110 ± 0.42
	Blood	5	5.01	100 ± 2.42
		100	97.1	97.1 ± 6.19
		180	188.1	105 ± 4.26
	DBS	5	4.73	94.6 ± 8.06
		100	101	101 ± 5.08
		180	160	88.7 ± 3.22
4 ºC (72 h, extracted)	Plasma	3	2.97	99.0 ± 3.62
		100	98.2	98.2 ± 1.73
		180	173	95.8 ± 0.28
	Blood	5	5.35	107 ± 4.04
		100	102	102 ± 5.88
		180	183	99.2 ± 0.83
	DBS	5	5.10	102 ± 5.60
		100	102	102 ± 8.44
		180	179	99.4 ± 8.61
-80 ºC (2–3 months)	Plasma	3	2.77	92.2 ± 1.56
		100	101	101 ± 0.65
		180	185	103 ± 1.56
	Blood	5	4.89	97.7 ± 15.5
		100	103	103 ± 7.13
		180	198	110 ± 19.0
	DBS	5	5.09	102 ± 3.00
		100	102	102 ± 5.86
		180	169	93.7 ± 6.37
Freeze/thaw cycles (3x)	Plasma	3	3.00	100 ± 0.92
		100	110	110 ± 2.29
		180	185	103 ± 1.83
	Blood	5	4.68	93.5 ± 0.88
		100	81.3	81.3 ± 22.1
		180	108	108 ± 1.96
	DBS	5	5.08	102 ± 7.04
		100	102	102 ± 0.93
		180	165	91.7 ± 5.39

[a] Values are means of *n* = 3 different human sources

The validation of *selectivity and sensitivity* was performed by analyzing six different blank and zero blank (without and with internal standard, respectively) human plasma and DBS samples and their corresponding LLOQs. Representative LC-MS/MS chromatograms of zero blank samples are illustrated in **figure 2** including the LLOQ of each matrix for comparison (892.5 → 307 m/z). Zero blank samples were extracted with the internal standard to exclude

that deuterated ivermectin partially contains ivermectin. No co-elution of unidentified peaks was observed proving the *selectivity* of the method and the purity of deuterated ivermectin (**figure 2**). The *sensitivity* of the method is defined by the LLOQ; 2 ng/mL and 3 ng/mL was achieved for plasma and DBS, respectively. The signal to noise ratio was at least 5:1 for the analytes as specified by the U.S. FDA guidelines for method's *sensitivity*.

Linearity of the method was tested in 2–200 ng/mL range for plasma and 3–200 ng/mL for DBS. Higher blood concentration of ivermectin was not expected due to earlier reported data of healthy volunteers.[13,15] 75% of calibration samples did not deviate more than ± 15% (LLOQ: ± 20%) from the nominal value. The correlation coefficient (r^2) of all validation experiments was > 0.997.

Accuracy and *precision* were tested in validation experiments. Intra-assay results were determined by analyzing three validation sets on one day and inter-assay experiments were performed with three validation sets on three individual days. In all sets, four QC concentrations were evaluated in six different human sources and compared to the calibration line (n_{tot} = 18 QC samples). At least 4/6 QC samples were in line with the regulations (± 15% of the nominal value, ± 20% for the LLOQ). Intra and inter assay results of the two matrixes (plasma and DBS) are similar with an accuracy of 96.3–101% and 96.1–107% and a precision of 3.79–12.2% and 4.89–12.2%, respectively (**table 4**). The consistent accuracy and precision < 15% prove the method's validity for the two matrixes. Moreover, varying hematocrits of DBS samples did not affect results.

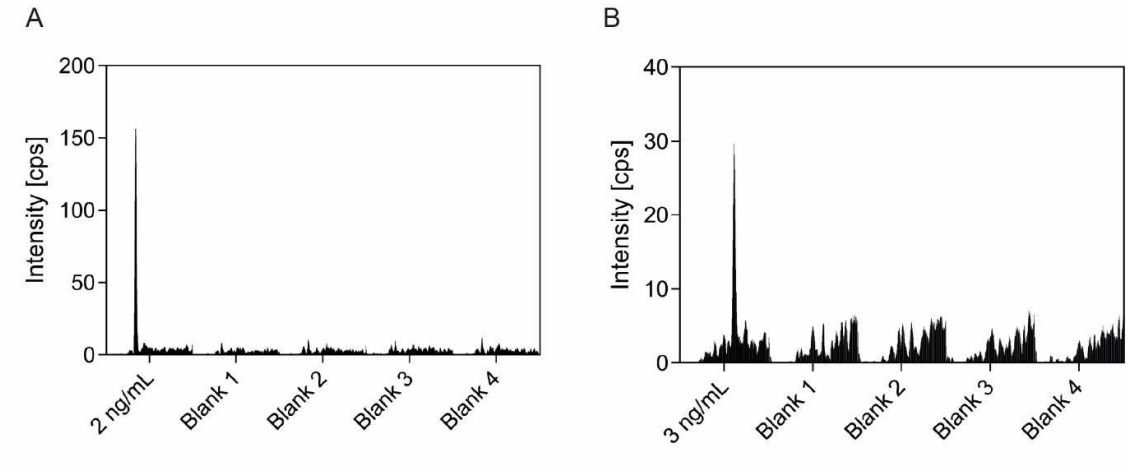

Figure 2. Background signal of four zero blank matrix samples: (A) plasma, (B) DBS. LC-MS/MS chromatograms of 892.5 → 307 m/z are presented when pure matrix was extracted. Internal standard was added to all zero blank samples to exclude interference of ivermectin detection by the internal standard. The chromatogram of the LLOQ is shown for comparison. The LLOQ of 2 ng/mL was achieved for plasma and 3 ng/mL for DBS.

Table 4. Intra- and inter-assay accuracy and precision of ivermectin extracted from plasma and DBS.

Matrix	$c_{nominal}$ [ng/mL]	Intra-assay[a]		Inter-assay[a]	
		$c_{ø}$ [ng/mL]	Accuracy ± CV [%]	$c_{ø}$ [ng/mL]	Accuracy ± CV [%]
Plasma	2	1.93	96.3 ± 8.24	1.93	96.6 ± 6.82
	3	2.97	99.1 ± 12.2	3.02	101 ± 8.20
	100	97.6	97.6 ± 7.43	99.1	99.1 ± 3.79
	180	177	98.1 ± 6.47	178	99.0 ± 4.07
DBS	3	3.13	104 ± 12.2	3.21	107 ± 11.7
	5	4.88	97.5 ± 10.6	4.80	96.1 ± 9.89
	100	98.2	98.2 ± 5.68	99.4	99.4 ± 4.89
	180	180	100 ± 8.08	179	99.6 ± 7.83

[a] Values are means of *n* = 18 QC samples of three independent validation sets.

The RRE and ME of ivermectin extracted from plasma and DBS were determined in three concentrations (low, middle and high) and results are illustrated in **table 5**. No ME were observed for the two matrixes with results > 88% and the coefficient of variation was low for DBS (< 6%) and plasma samples (< 11%).

RRE of ivermectin resulted in acceptable values for plasma (> 77%) with low deviation. RRE of DBS yielded in consistent but rather low results (47–56%). The low RRE might be caused by the extraction procedure, where two spots are extracted in one Eppendorf Tube®. The two DBS spots tended to stick together during extraction so that the solvents possibly could not reach effectively both sites of both spots. For future studies, the extraction procedure should be further optimized to increase RRE results.

Table 5. Relative recovery (RRE) and matrix effect (ME) of ivermectin extracted from plasma and DBS.

Matrix	$c_{nominal}$ [ng/mL]	RRE ± CV [%][a]	Mean ± CV [%][b]	ME ± CV [%][a]	Mean ± CV [%][b]
Plasma	3	85 ± 13	81 ± 9.2	97 ± 11	92 ± 8.3
	100	77 ± 7.6		91 ± 6.5	
	180	81 ± 2.2		88 ± 1.3	
DBS	5	56 ± 1.5	52 ± 3.4	102 ± 3.75	106 ± 4.88
	100	47 ± 6.2		107 ± 1.83	
	180	53 ± 8.2		107 ± 6.18	

[a] Values are means of *n* = 4 different human sources

[b] Values are means of *n* = 12 samples (3 concentrations measured in 4 human sources)

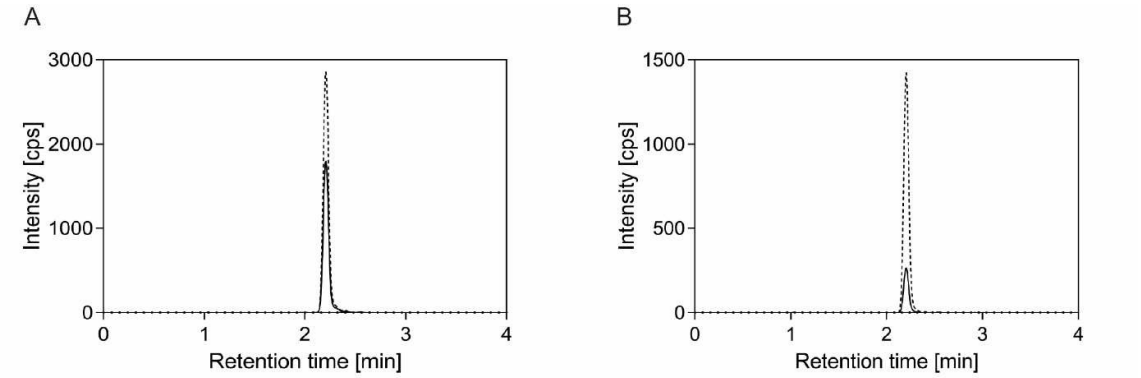

Figure 3. Representative LC-MS/MS chromatograms of ivermectin (892.5 → 307 m/z, black line) with internal standard (dashed line) extracted from (A) plasma and (B) DBS samples from the identical patient 4 h post-treatment.

3.3 Method application

The LC-MS/MS method was applied to samples collected from 11 *T. trichiura* infected adult volunteers treated with 200 µg/kg ivermectin in a clinical study in rural Côte d'Ivoire. Ivermectin was extracted from plasma and DBS samples and representative LC-MS/MS chromatograms of ivermectin are shown in **figure 3**. The concentration-time profiles (**figure 4A**) illustrate that the time to reach maximum ivermectin concentration is identical for plasma and DBS samples (t_{max} = 3.9 h, **table 6**). A slow clearance and thus a long $t_{1/2}$ (26 and 32 h, respectively) was observed for plasma and DBS samples as ivermectin was still detectable after 72 h post-treatment. The detected median of the maximal ivermectin concentration (C_{max}) was 51.6 ng/mL (26.7–91.2 ng/mL) and 40.1 ng/mL (22.6–64.3 ng/mL) for plasma and DBS samples, respectively (**table 6**). The evaluated AUC is 987 ng/ml*h for plasma and 810 ng/ml*h for DBS samples. No statistical significant difference was observed between the PK parameters of the two matrices. Moreover, Bland-Altman analysis was performed to evaluate the statistical deviation between the quantified ivermectin concentrations in plasma and DBS samples (**figure 4B**). Consistent and good agreement was obtained of plasma/DBS samples since most data are within confidence intervals (± 95%).

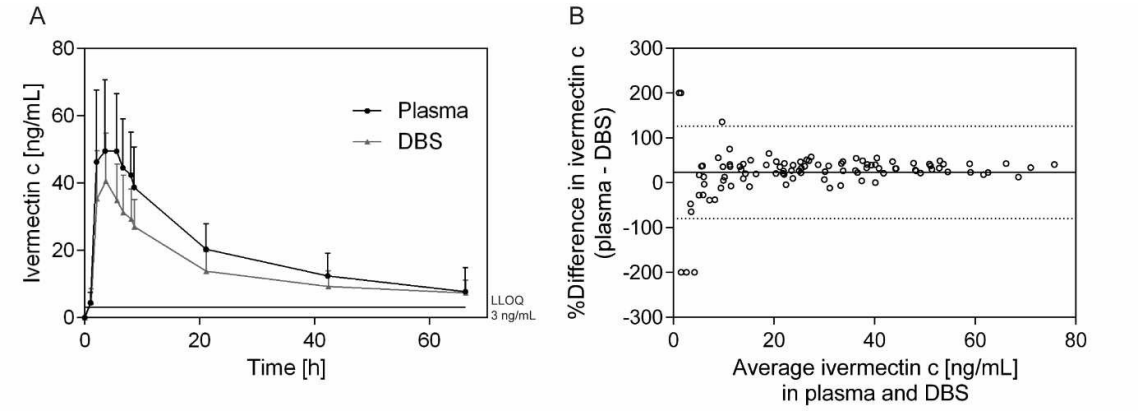

Figure 4. (A) Concentration time profile of ivermectin extracted from plasma and DBS. LLOQ for DBS (3 ng/mL) is shown as a black line. (B) Bland-Altman analysis comparing results of plasma and DBS clinical trial samples. 95% confidence intervals are shown as dashed lines. Bias is illustrated as a black line.

Table 6. Pharmacokinetic parameters of ivermectin extracted from plasma and dried blood spots (DBS). No statistical significant difference between parameters derived from plasma and DBS samples was reported ($p < 0.05$).

Matrix		$t_{1/2}$ [h]	t_{max} [h]	C_{max} [ng/mL]	AUC [ng/ml*h]
Plasma	Median	26.2	3.9	51.6	987
	Min	12.1	2.1	26.7	511
	Max	66.1	9.1	91.2	2199
DBS	Median	32.3	3.9	40.1	810
	Min	17.8	2.3	22.6	348
	Max	84.2	9.2	64.3	1664

4. Conclusion

A rapid, robust and sensitive LC-MS/MS method was established to quantify ivermectin extracted from DBS and plasma. Accuracy, precision, selectivity and sensitivity fulfilled the requirements defined by U.S. FDA for DBS, plasma and blood samples, but ivermectin was proven stable under several conditions only in DBS and plasma. Thus, the method validation was accomplished with consistent results for plasma and DBS but partially failed for blood samples. When the method was applied to PK clinical trial samples from *T. trichiura* infected adults, identical t_{max} and similar C_{max}, $t_{1/2}$ and AUC were obtained for plasma and DBS samples. DBS should therefore be the matrix of choice for future clinical trials, especially when children are volunteering. This allows a more ethical and less invasive procedure for patients and an easier and consistent technique for field and laboratory work.

5. Conflict of interest

There is no conflict of interest to declare.

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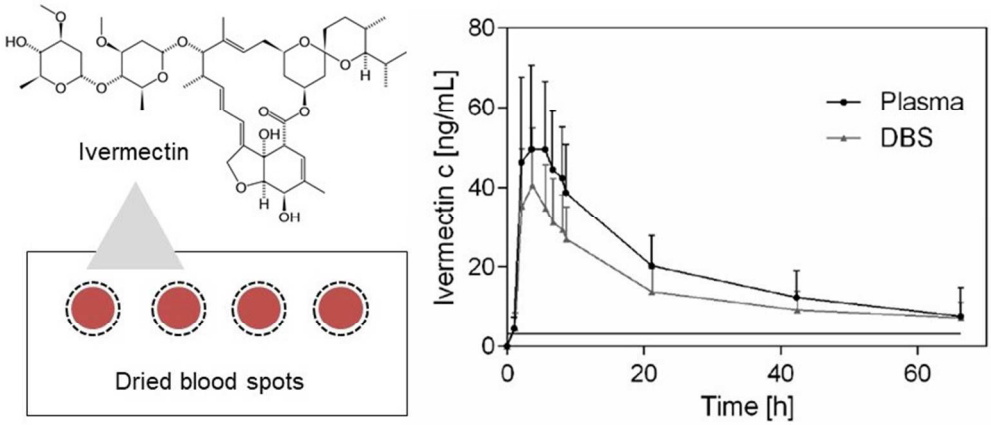
Ivermectin was quantified in dried blood spot samples derived from *Trichuris trichiura*-infected adults with a validated LC-MS/MS method.



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