

Article

Simultaneous LC-MS/MS Method for the Quantitation of Probenecid, Albendazole, and Its Metabolites in Human Plasma and Dried Blood Spots

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Highlights:

What are the main findings?

- The validated method was robust, reliable, and sensitive for quantifying ABZ, ABZ-OX, ABZ-ON, and PR simultaneously from the plasma and dried blood spots.
- The method is well suited for therapeutic drug monitoring due to its high sensitivity, minimal sample volume requirement, and the ability for remote collection.

What is the implication of the main finding?

- Dried blood spot (DBS) analysis represents an alternative sampling technique over conventional blood-sampling methods, particularly for sample collection, enabling a non-clinical setup without requiring special equipment or trained healthcare personnel. Additionally, it minimizes the solvent volume and analysis time and reduces transportation and storage needs.



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Abstract: Millions of individuals throughout the world suffer from lymphatic filariasis (LF), which is a morbid disease caused by *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*. These infections belong to tissue-invading nematodes and are one of the major neglected tropical diseases that often result in permanent and enduring disability among individuals in endemic regions. Due to combination therapy, LF eradication has drastically decreased infections globally. The development of blood micro-sampling techniques allowing precise quantitation of drugs in blood would facilitate pharmacokinetic (PK) studies in remote populations. Therefore, an LC-MS/MS bioanalytical method was utilized to analyze albendazole (ABZ), albendazole sulfone (ABZ-ON), albendazole sulfoxide (ABZ-OX), and probenecid (PR) in plasma and dried blood spots. Solid-phase extraction was utilized to extract the analyte from both plasma and blood-spiked DBS. Analytes of interest were eluted with a gradient mobile system using 0.05% formic acid in water (A) and 0.05% formic acid in methanol (B) and separated using a reversed-phase Acquity[®] BEH C18 UPLC column (100 × 2.1 mm, 1.7 μm). Precision and accuracy at each QC level were within the acceptable limit, i.e., ±15% for all analytes in both the matrices. Tests for stability under laboratory and storage conditions indicated that no notable changes were observed for plasma and DBS. The LC-MS/MS method demonstrated its capability to consistently identify all target analytes (ABZ, ABZ-ON, ABZ-OX, and PR) at low concentrations, even at the small specimen volumes obtained from DBS cards. This confirms the efficacy and durability of DBS cards as a micro-sampling technique. Moreover, it enhances collection efforts for therapeutic drug monitoring in remote locations for patients infected with lymphatic filariasis.

Keywords: dried blood spot; UPLC-MS/MS; lymphatic filariasis; ABZ; ABZ-OX; ABZ-ON

1. Introduction

Traditional pharmacokinetic studies typically rely on analyzing drug concentrations in plasma samples. However, recent advancements have explored alternative sample matrices, such as dried blood spots (DBS), which offer distinct advantages in terms of ease of collection, stability, and feasibility of transportation and storage. DBS involves the application of a small volume of blood onto a filter paper, which is subsequently dried and stored for analysis. The use of DBS for pharmacokinetic studies has gained increasing recognition due to its minimally invasive nature, reduced sample volume requirements, and improved stability of analytes during storage and transportation. Furthermore, DBS has been shown to provide reliable and accurate measurements of drug concentrations, making it a promising tool in pharmacokinetic research. Several studies have already explored the utility of DBS in pharmacokinetic analyses for various drugs. DBS collection was used to investigate the simultaneous analysis of seven antimalarial drugs and metabolites in clinical studies [1]. DBS has been used to assess vitamin D status in patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [2,3].

Albendazole (ABZ), a benzimidazole derivative broad-spectrum anthelmintic widely employed against various helminthic infections caused by intestinal worms and tissue-dwelling parasites, was introduced to treat humans for parasitic infections in 1982 [4,5]. ABZ is extensively metabolized in the liver and primarily catalyzed by cytochrome P450 enzymes into ABZ-OX, which is further oxidized to produce albendazole sulfone ABZ-ON, an active metabolite that exhibits superior anthelmintic properties compared to the parent compound [6].

To date, drugs such as ivermectin (IVM), diethylcarbamazine (DEC), and ABZ are administered in combination as part of mass drug administration (MDA) programs for the treatment and control of lymphatic filariasis (LF) [7,8]. The combination of IVM/DEC/ABZ in a single treatment shows a promising effect on LF elimination, as reported by Thomsen et al. (2016) [9]. However, the combination of these drugs poses a severe adverse impact in the condition where LF coexists with *Oncho/Loa loa* [9,10]. Furthermore, under certain conditions, MDA will not be able to eliminate transmission completely [11,12]. Therefore, it is crucial to identify alternative therapies capable of delivering a reliable macrofilaricidal effect to reduce LF transmission effectively.

Several research articles have thoroughly elucidated and documented the symbiotic interaction between *Wolbachia* and the filarial worm [13–15]. Some researchers have suggested that chemotherapy might be a possible target for *Wolbachia* lymphatic filariasis [14,15]. In vitro study of probenecid (PR) has shown promising macrofilaricidal effects against *B. malayi* worms and suggests that probenecid might be a possible potential microfilaricide for humans [16]. Flynn et al. 2019, observed that probenecid exhibits a synergistic macrofilaricidal effect with albendazole against adult *B. malayi* worms [16].

To date, several analytical methods, including high-performance liquid chromatography (HPLC) with ultraviolet detection (UV) [17,18], fluorescence detection [19], and capillary electrophoresis [20] have been reported to determine ABZ and its metabolites. Furthermore, some LC-MS/MS methods [21,22] have been used to quantify ABZ and its metabolites. However, most of these methods utilize protein precipitation [18] and liquid-liquid extraction [23]. A few articles have used the solid-phase extraction [24] method. Our group previously published a similar article, but that study involved a different combination of drugs, moxidectin and ivermectin, with albendazole and its metabolite combined with diethylcarbamazine in human plasma [25]. However, no one has reported an LC-MS/MS method for simultaneously analyzing ABZ, ABZ-OX, ABZ-ON, and PR in plasma and dried blood spots. We report here the full LC-MS/MS method for the quantitation of these drugs in plasma and DBS collection cards.

The objective of this study is to develop a new, simplified, and efficient methodology for quantifying accurately ABZ, ABZ-OX, ABZ-ON, and PR in plasma and dried blood spot matrices by utilizing LC-MS/MS. By comparing sample recovery and matrix effects, this research evaluates the suitability and reliability of DBS as a viable alternative to conven-

tional blood-sampling methods. Previous studies have reported the feasibility of utilizing DBS collection for quantifying albendazole and metabolites [26]. This study builds on these findings and includes the assessment of PR as part of a potential therapeutic regimen for preventing LF transmission. Our goal was to establish an accurate, robust, and reliable extraction and bioanalytical LC-MS/MS approach for determining the concentration of ABZ, ABZ-OX, ABZ-ON, and PR in DBS for comparison with methods utilizing plasma samples. These findings will facilitate sample collection in remote locations and form the foundation for evaluating the correlation between drug levels and the effectiveness of LF-MDA regimens.

2. Materials and Method

2.1. Chemical and Materials

Albendazole (ABZ, purity, $\geq 98\%$), albendazole sulfoxide (ABZ-OX, purity, $\geq 99.9\%$), albendazole sulfone (ABZ-ON, purity, $\geq 99.6\%$), oxibendazole (OBZ, purity, $\geq 99.9\%$) probenecid (PR), and ABZ-d3 (purity, $\geq 98\%$) (Figure 1) were procured from Sigma-Aldrich, St. Louis, MO, USA. LC-MS grade acetonitrile, formic acid, and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ultrapure water (TDW) was produced in-house using a Barnstead Genpure water-purification system (Thermo Scientific, Waltham, MA, USA). Drug-free human blood was procured from ZenBio (Durham, NC, USA). Whatman DBS collection cards (Whatman®) were procured from Cytiva (Little Chalfont, UK). We procured commercially available pooled human plasma from Innovative Research (Peary Court Novi, MI, USA), which used K2EDTA as an anticoagulant. Agilent bond elute 96-well plates (C18, 50 mg per 1 mL) and Agilent bond elute cartridges (C18, 50 mg per mL) were purchased from Agilent (Santa Clara, CA, USA). All other reagents used in the study were of analytical quality and procured from reputable commercial vendors.

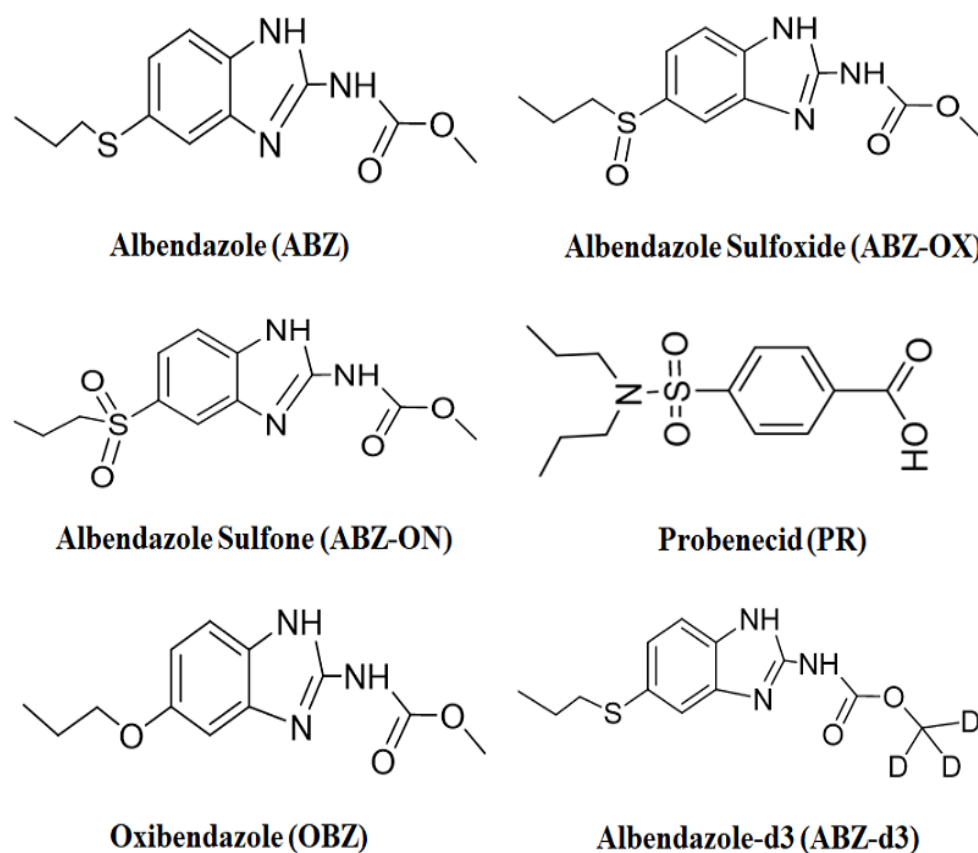


Figure 1. Chemical structures of ABZ, ABZ-OX, ABZ-ON, PR, OBZ, and ABZ-d3.

2.2. Preparation of Calibration Curve, QC Samples in Plasma, and DBS Card Spiked with Blood

The stock solution of ABZ, ABZ-OX, ABZ-ON, PR, OXZ (IS), and ABZ-d3 (IS) was prepared in methanol with a final concentration of 1 mg/mL. Two separate stock solutions were constructed for calibration standards (CCs) and quality-control (QCs) samples, respectively. The stock solutions were further diluted with MeOH to create various mixed working standards (MWS). The MWS was used to generate the calibration curve (CC) and quality-control (QCs) samples. The stocks, MWS, and QCs spiking solutions were stored at -20°C until required or freshly prepared as necessary.

For the plasma, the calibration standards were created by adding 10 μL of a $10\times$ higher concentration MWS solution into 90 μL of human plasma, resulting in the concentration range of 1–2000 ng/mL for PR, 0.5–1000 ng/mL for ABZ-OX, and 0.1–200 ng/mL for ABZ and ABZ-ON [23,25,27]. Quality-control samples at four different concentrations (1, 5, 500, and 1500 ng/mL for PR; 0.5, 2, 200, and 750 ng/mL for ABZ-OX; 0.1, 0.5, 40, and 150 ng/mL for ABZ and ABZ-ON), lower limit of quantification (LLOQ), low-quality control (LQC), middle-quality control (MQC), and high-quality control (HQC), were prepared in three replicates, distinct from the calibration standards. Each calibration and QC sample was adjusted to a final volume of 100 μL in plasma. Additionally, we added 10 μL of IS spiked into all CC and QC solutions.

For the DBS samples, the final concentration of each drug in the DBS card for the calibration curve and QCs sample solutions was determined by adding 2.5 μL of a $40\times$ higher concentration MWS solution to 97.5 μL of EDTA containing human blood, resulting in achieving concentration ranges of 0.5–1000 ng/mL for ABZ-OX, 0.1–200 ng/mL for ABZ and ABZ-ON, and 1–2000 ng/mL for PR. QCs samples at four concentration levels, viz., (LLOQ, LQC, MQC, and HQC) for (1.5, 3, 200, and 750 ng/mL for ABZ-OX, 0.3, 0.6, 50, and 150 ng/mL for ABZ and ABZ-ON, 3, 6, 500, and 1500 ng/mL for PR), respectively. Each QC sample was prepared in three replicates.

Spiked blood was mixed by gentle pipetting, and 40 μL of the blood was then applied to Whatman paper[®] cards using a micropipette. The DBS cards were air-dried for at least 2 h. After drying, the DBS card was sealed inside a plastic bag and stored at -80°C for further analysis and quantitation.

2.3. Samples Preparation and Extraction from Plasma and DBS Card

To 90 μL of plasma, 10 μL of MWS ($10\times$) and 10 μL of IS were vortexed for 30 s and further diluted with 500 μL of 0.2% formic acid, mixed for 30 s, and then transferred onto Agilent bond Elute 96-well plate solid-phase extraction (SPE) cartridges pre-conditioned with MeOH (1 mL), followed by 0.2% formic acid (1 mL). Afterward, the cartridges containing drugs were washed with 2 mL of MeOH (5%) and eluted with 1 mL of MeOH containing 1% formic acid (FA), followed by 1 mL of 80% acetonitrile. For both standards and samples, the eluates were dried under vacuum conditions at a 45°C temperature and dissolved in 200 μL of a solution containing 0.1% FA and MeOH in the ratio of 60:40 (v/v). Further, the reconstituted solution was centrifuged and transferred to 96-well plates for injection into the LC-MS/MS.

For the DBS samples, a 4 mm $\times$ 4 diameter punch was performed for every spot by using a DBS automatic machine BSD Duet device (Model No. BSD600/DUET, BSD Robotics Brendale, Australia) into a 96-well plate. Fifty μL of TDW were added and vortexed for 10 min. Then, a further 150 μL of a mixture of acetonitrile and water in a 4:1 (v/v) ratio containing 0.1% formic acid was added, and again vortexed for 10 min. Afterward, 10 μL of IS were spiked in all the DBS samples. The sample was mixed with 500 μL of 0.1% formic acid and subjected to sonication for 30 min. After sonication, the plates were centrifuged at 3500 rpm for 10 min, and the supernatant was separated and applied onto SPE cartridges (96 well plates) that had been pre-conditioned with 1 mL of methanol, followed by 1 mL of solution containing 0.2% formic acid. The cartridges containing the samples were rinsed with 2 mL of 5% MeOH and then eluted with 1 mL of a solution containing 1% FA in MeOH, followed by 1 mL of 80% acetonitrile. The eluted solutions of CC and QCs were evaporated

using nitrogen gas under vacuum conditions at 45 °C temperature and dissolved in 150 µL of a mixture comprising 0.1% formic acid and methanol (in the ratio of 60:40, *v/v*). Further, the reconstituted solution was centrifuged at 3500 rpm for 5 min and transferred to 96-well plates for injection into the LC-MS/MS.

2.4. Instrumentation and Chromatographic Conditions

The analysis was performed using a UPLC-MS/MS 8050 system (Shimadzu Scientific Instruments, Columbia, MD, USA) that consisted of a UPLC Nexara system equipped with two LC-30 AD pumps, a CTO-30AS column oven, a SIL-30AC autosampler, a CBM-30 elite controller, and a DGU-30AC degassing unit, with a DUIS interface that contained electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) (Shimadzu Scientific Instruments, Columbia, MD, USA).

The chromatographic separation was achieved with a UPLC C18 column (Acquity UPLC® BEH; 1.7 µm, 100 × 2.1 mm, Waters, Milford, MD, USA) having a Security Guard Cartridge (Acquity UPLC C18, Waters, Milford, MD, USA). The mobile phase was prepared by using ultra-pure water with 0.05% formic acid (mobile phase A) and methanol with 0.05% formic acid (mobile phase B), with a flow rate set at 0.2 mL/min. The chromatographic elution was accomplished through gradient elution, and the conditions were as follows. Phase B was initiated at 35% during the first minute of the run and gradually increased to 70% at 6 min. Subsequently, it was further raised to 95% at the 8th minute and held for 1 min at 95% of B, followed by a re-equilibration condition set to 35% B within 0.1 min, which was maintained for three additional minutes to allow column equilibration. The injection volume was set to 2 µL for plasma samples and 5 µL for DBS samples. The mass spectrometry system was conducted in the multiple reaction monitoring (MRM) modes with unit resolution. The mass-spectrometer parameters, such as temperature, voltage, and gas pressure, were optimized for each compound through auto-method optimization via product ion search using a 1 µg/mL solution in methanol for both analytes and the internal standard (IS). Analytes were detected in the positive ESI ionization with specific mass-spectrometer source settings: nebulizer gas at 2.0 L/min, heating gas at 10 L/min, drying gas at 10 L/min, interface temperature at 375 °C, desolvation line temperature at 250 °C, and heat block temperature at 300 °C. The MRM transitions and the corresponding optimum MS parameters (Q1, Q3 voltage potential, and collision energy) for each analyte and IS are provided in Table 1. LabSolutions LC-MS Ver. 5.99 sp2. Software (Shimadzu Scientific Inc., Columbia, MD, USA) was employed for data acquisition and quantitation.

Table 1. Summary of MS/MS parameters: precursor ion, fragment ions, voltage potential (Q1), collision energy (CE) and voltage potential (Q3), retention time (RT), of ABZ, ABZ-OX, ABZ-ON, PR, OXZ (IS), and ABZ-d3 (IS).

Analytes	MRM Transition <i>m/z</i> (Q1 → Q3)	Q1 (V)	CE (V)	Q3 (V)	RT
ABZ	266.10 > 234.40	−28	−20	−26	6.7
ABZ-OX	282.10 > 240.35	−30	−14	−26	3.7
ABZ-ON	298.10 > 159.30	−15	−35	−17	4.2
PR	286.10 > 202.05	−29	−17	−22	8.1
OXZ	250.20 > 176.40	−29	−27	−19	4.8
ABZ-d3	269.40 > 234.4	−26	−19	−28	6.7

2.5. Validation Studies

The bioanalytical method was validated as per the guidance for industry bioanalytical method validation guidelines [28]. The validation parameters include recovery, linearity, selectivity, precision, accuracy, and stability.

2.5.1. Recovery, Matrix Effect, Selectivity, and Specificity

For plasma and DBS samples, to estimate the recovery, three different sets of samples were employed, i.e., “normal”, “post extracted”, and “neat QCs”. The “normal” QC samples were prepared by extracting blood spiked onto the DBS card or blank plasma, following the procedure outlined in Section 2.2. “Post-extracted” QCs were prepared by adding drug-free blood on a DBS card or drug-free plasma. The “Neat QCs” sample is just a diluted working stock in the mobile phase. The sample was produced in triplicate for each QC level, i.e., LQC, MQC, and HQC. The recovery of each analyte was determined by comparing the response from the peak areas of pure QC samples to that of the post-extracted QCs, and the result was expressed as a percentage.

After injecting blank DBS or blank plasma extracted samples, the matrix effects were evaluated qualitatively by a post-column infusion of each analyte and IS (1 µg/mL). The LC-MS/MS signal was visually observed in order to identify any interference peak at the retention time of the analytes and IS. Additionally, the matrix effect was determined quantitatively by dividing the peak area of the post-spiked samples at the three QC levels, i.e., LQC, MQC, and HQC, by the peak area of the neat standards and then multiplied by 100 at each corresponding concentration.

2.5.2. Linearity and Lower Limit of Quantification

For plasma and DBS samples, linearity was assessed by analyzing the plasma or DBS card spiked with an analyte within the concentration range specified in Section 2.2 and using the least squares method. Each calibration curve included a blank sample, a zero sample (blank + IS), and ten non-zero concentrations. Linear regression analysis was applied to fit the results using a weighting factor of $1/X^2$.

The accuracy of the calibration points for both the plasma and DBS samples needed to fall within the range of 85–115% of the expected concentration, except for the LLOQ, where accuracy was acceptable $\pm 20\%$. The correlation coefficient (r^2) was required to be ≥ 0.99 for all analytes.

The % bias of accuracy and % R.S.D of precision for all QC levels of each matrix, viz. plasma and DBS, lies within $\pm 15\%$ of each concentration.

2.5.3. Sensitivity and Carryover

The sensitivity of the validated method was assessed using the signal-to-noise ratio (S/N) obtained from the response of the analyte in the calibration standards. An S/N ratio of ≥ 3 was required to set the limit of detection (LOD), and an S/N ratio of ≥ 10 for all the QC levels, except for LLOQ, which should be greater than 5. The calibration curves were generated by plotting the peak-area ratio against the concentration for all analytes for both plasma and DBS.

To quantify the carryover, blank samples were directly injected following the upper limit of quantification (ULOQ), and the peak area of the blank was analyzed. The peak area in a blank sample lower than 20% of the LLOQ should be considered negligible carryover.

2.5.4. Dilution Integrity

The impact of dilution was examined to confirm that the samples could be diluted with blank plasma without altering the concentration of ABZ, ABZ-OX, ABZ-ON, and PR. Plasma samples spiked with a concentration of 400 ng/mL for ABZ and ABZ-ON, 2000 ng/mL for ABZ-OX, and 4000 ng/mL for PR were diluted with blank plasma at dilution factors of 2 and 5. As a validation requirement, these replicates needed to meet both precision criteria ($\leq 15\%$) and accuracy ($100 \pm 15\%$), like other quality-control (QCs) samples.

2.5.5. Precision and Accuracy

Accuracy and precision were evaluated for intra- and inter-day variation using triplicate QC samples at four concentration levels (LLOQ, LQC, MQC, and HQC) generated on

the same day. Three sets of replicates were prepared for each QC level and analyzed within a single day. Additionally, three sets of replicates were prepared for each QC level and analyzed on three separate days to evaluate intra- and inter-day precision and accuracy. To ensure reliable results, each set of triplicates should contain at least two samples falling within the $\pm 15\%$ bias of the QC concentration for all analytes, except LLOQ, where this range can extend up to 20%. Furthermore, the relative coefficient of variation should remain below 15% for all analytes, except for the LLOQ, where it should be lower than 20%.

2.5.6. Stability

For the plasma, the autosampler stability at 4 °C for 36 h was investigated for ABZ, ABZ-OX, ABZ-ON, and PR in plasma. Bench-top stability was assessed at room temperature (20 °C) for 8 h using QC samples in three replicates. Additionally, storage stability was also assessed for 90 days at -80 ± 5 °C. Freeze–thaw stability was assessed after subjecting the samples to three freeze–thaw cycles (room temperature to -80 ± 5 °C).

For the DBS samples, the autosampler stability of ABZ, ABZ-OX, ABZ-ON, and PR in the DBS spot was evaluated at 4 °C for 36 h. The DBS samples were kept on the bench-top at an ambient temperature (20 °C) over 45 days before being processed and analyzed to assess the benchtop stability, using all sets of QC samples in replicates. Additionally, the storage stability was evaluated at a temperature of -80 ± 5 °C for 365 days. The QC samples were processed as outlined in the previous Section 2.3 and stored in a sealed plastic bag with a desiccant package. All stability studies were conducted in three replicates for three QC levels, viz: LQC, MQC, and HQC, for both the plasma and DBS samples.

3. Result and Discussion

3.1. LC-MS/MS Method Development and Chromatographic Conditions Optimization

Different combinations of mass spectrometric conditions and chromatography were adjusted for optimization during method development for all analytes. The positive ESI ionization mode was chosen for MS detection as a better MS response for the analytes and IS than the positive mode of APCI. Under the optimized chromatographic conditions, the full Q1 MS spectra scan showed that the protonated precursor $[M + H]^+$ ions at specific m/z values for ABZ, ABZ-OX, ABZ-ON, PR, OXZ, and ABZ-d3: 266.1, 282.1, 298.1, 286.1, 250.2, and 269.4 respectively. The fragmentation of these analytes and the IS was determined using an auto-optimized method through precursor-ion search, with a stock solution for each analyte at a concentration of 1 µg/mL, as previously documented in our report article [27]. The precursor > product ions observed for ABZ, ABZ-OX, ABZ-ON, PR, OXZ, and ABZ-d3 had specific m/z values: 266.10 > 234.40, 282.10 > 240.35, 298.10 > 159.30, 286.10 > 202.05, 250.20 > 176.40, and 269.40 > 234.41, respectively. Subsequently, compound-dependent parameters, including voltage potential Q1 (V) and Q3 (V), along with collision energy (CE), were systematically optimized to achieve the maximum signal intensity for all analytes and the IS (Table 1).

Chromatographic conditions were evaluated on a C18 column of different length ranges from 50–150 mm with different diameters. Different combinations of mobile phase systems, such as water, ammonium acetate, acetic acid, or formic acid with water and ammonium, format with methanol or acetonitrile with and without formic acid. We first tried isocratic elution for analyte separation with different flow rates, but it was insufficient to separate all the analytes. So, we further move with the gradient elution method with water and methanol with 0.05% formic acid. The gradient method with 0.05% formic acid in water and methanol serves as the best mobile phase for the elution of all the analytes. The analytes and IS resolution were achieved on an Acquity UPLC® BEH C18 column (1.7 µm, 100 × 2.1 mm) equipped with an Acquity UPLC C18 guard column (Waters, Milford MD) with the 0.05% formic acid in water and methanol, respectively, at a flow rate of 0.2 mL/min. The column temperature was maintained at 40 °C. ABZ-d3 was utilized as an IS for analyzing ABZ, ABZ-ON, ABZ-OX, and OBZ as an IS for PR. No interference of endogenous compound chromatograms was observed when analyzing

blank DBS samples at the retention time of the analytes of ABZ, ABZ-OX, ABZ-ON, and PR (Figure 2). Additionally, post-column infusion confirmed the absence of matrix effects, as no signal amplification or diminishment was observed at the retention time of ABZ, ABZ-OX, ABZ-ON, and PR.

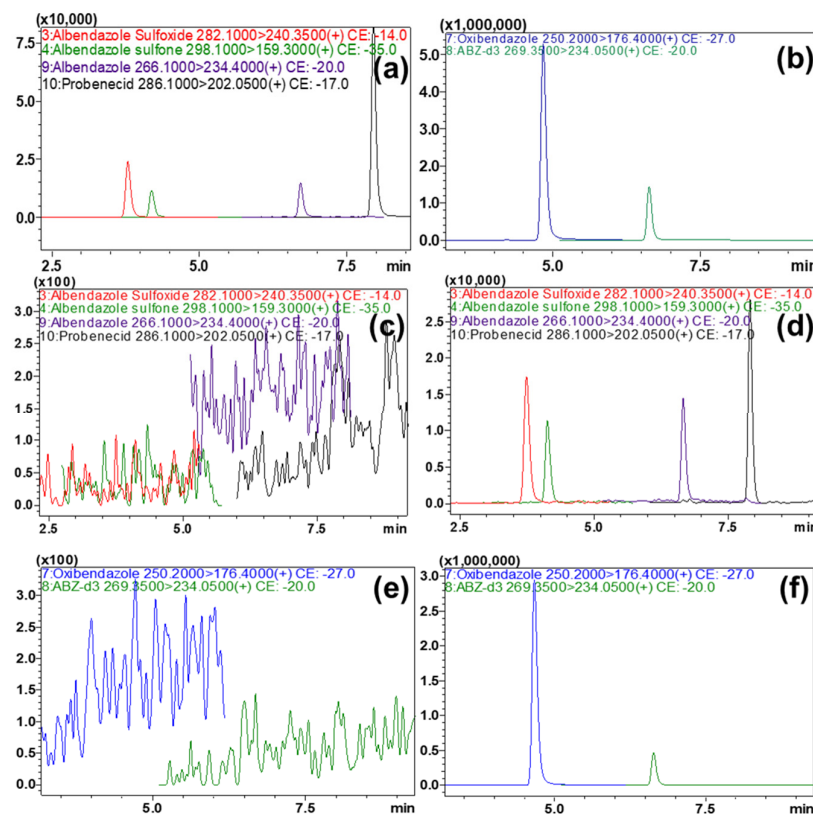


Figure 2. Representative MRM ion-overlay chromatograms of ABZ, ABZ–OX, ABZ–ON, PR, OXZ, and ABZ–d3 (a) standard spiked at LQC level in neat solvent (b) IS spiked at in neat solvent (c) extracted blank DBS (d) standard spiked at LQC level extracted from DBS (e) extracted Blank DBS without IS (f) spiked IS extracted from DBS.

3.2. Specificity and Selectivity

The specificity of the method was validated for all the analytes by extracting and analyzing blank samples from different sources, both with and without IS. The chromatogram of the blank sample demonstrated that there was no co-elution of unidentified molecules from the matrices. In Figure 2, the chromatogram overlay depicts the analysis of extracted blank DBS and analytes spiked at an LQC concentration in DBS. Notably, no interfering peaks are observed at the retention times of the analytes and IS. The relative standard deviation (R.S.D.) for these measurements falls within the acceptable limit of $\pm 5\%$, as required by the US FDA guidelines. The retention time for ABZ, ABZ-OX, ABZ-ON, PR, OXZ, and ABZ-d3 was 6.7, 3.7, 4.2, 8.1, 4.8, and 6.7 min, respectively.

3.3. Accuracy and Precision

The accuracy and precision were assessed in both intra- and inter-day for four QC levels and summarized in Table 2a,b for both the plasma and DBS cards. The result indicates that the bioanalytical method is precise, with bias ranging from 0.8–15% for both plasma and DBS. The bias falls within the acceptable limits of $\pm 15\%$ for all QC levels except LLOQ, where it was $\pm 20\%$. The precision (% R.S.D) values for the intra- and intra-day indicate good assay precision, ranging from 0.6 to 14.9% for both plasma and DBS. Based on the accuracy and precision data, it can be concluded that the validated method exhibits sufficient accuracy and precision for quantitating all the analytes in any clinical setting.

Table 2. (a) Intra- and inter-day accuracy (% bias) and precision (% R.S.D.) of ABZ, ABZ-OX, ABZ-ON, and PR in human plasma. (b) Intra- and inter-day accuracy (% bias) and precision (% R.S.D.) of ABZ, ABZ-OX, ABZ-ON, and PR in human blood spiked at DBS.

(a)					
Analytes	QCs Levels	Accuracy (% Bias)		Precision (% R.S.D)	
		Intra-Day (% Bias)	Inter-Day (% Bias)	Intra-Assay (% R.S.D)	Inter-Assay (% R.S.D)
ABZ	LLQO	4.8	6.7	10.8	12.9
	LQC	−3.1	10.6	1.1	14.9
	MQC	−5.4	−4.2	1.1	5.1
	HQC	4.0	9.7	6.9	5.9
ABZ-OX	LLQO	4.8	13.1	7.6	8.4
	LQC	−14.9	−11.5	1.1	4.4
	MQC	9.3	12.6	0.6	5.5
	HQC	−8.3	−9.2	0.5	4.1
ABZ-ON	LLQO	2.1	7.3	0.5	13.3
	LQC	1.8	13.6	9.6	12.8
	MQC	2.1	8.8	8.4	5.0
	HQC	12.7	11.9	0.9	9.1
PR	LLQO	7.0	7.4	4.1	4.4
	LQC	−10.3	1.7	5.7	13.5
	MQC	−9.6	−7.1	3.1	5.3
	HQC	−1.4	4.2	2.4	8.3
(b)					
Analytes	QCs Levels	Accuracy (% Bias)		Precision (% R.S.D)	
		Intra-Day (% Bias)	Inter-Day (% Bias)	Intra-Assay (% R.S.D)	Inter-Assay (% R.S.D)
ABZ	LLQO	−7.4	−5.2	11.2	9.5
	LQC	−2.4	−6.6	8.7	6.6
	MQC	−4.9	−5.6	3.3	5.0
	HQC	13.2	14.6	2.5	6.2
ABZ-OX	LLQO	−2.3	1.8	5.6	7.4
	LQC	−7.2	−3.7	11.9	13.4
	MQC	17.7	2.6	6.0	12.0
	HQC	−6.2	−3.9	10.5	12.9
ABZ-ON	LLQO	−6.1	4.0	−4.6	7.6
	LQC	−5.8	7.0	9.2	13.2
	MQC	−7.0	8.8	0.6	10.2
	HQC	3.8	5.3	−5.2	7.7
PR	LLQO	6.5	−2.9	5.3	11.9
	LQC	0.8	2.8	9.4	6.5
	MQC	−1.0	2.7	9.1	9.8
	HQC	9.2	4.3	13.0	6.8

3.4. Recovery and Matrix Effect

The recovery and matrix effect of analytes was evaluated by comparing the analytical results for extracted samples at LQC, MQC, and HQC concentrations for each analyte in both matrices, i.e., plasma and DBS, as summarized in Table 3a,b. The % coefficient of variation (CV) and mean recoveries at these concentration levels were consistent, precise, and reproducible, and the values fell within the permissible range of $\pm 15\%$. As per US FDA guidelines, the recovery of analytes does not need to be 100%; however, it should exhibit a consistent level of recovery [28]. Furthermore, the mean extraction recovery of the IS was $81.2 \pm 2.7\%$ and $62.5 \pm 4.6\%$ for OBZ and ABZ-d3, respectively. The matrix effects were found to be 87–105%. Table 3a,b indicated minimal ion suppression or enhancement from human plasma or DBS under the present conditions.

Table 3. (a) Mean extraction recoveries and matrix effect of the ABZ, ABZ-OX, ABZ-ON, and PR from plasma. (b) Mean extraction recoveries and matrix effect of the ABZ, ABZ-OX, ABZ-ON, and PR from DBS.

(a)						
Analytes	Mean Extraction Recovery (%) (Mean $\pm$ SD, n = 3)			Mean Matrix Effect (%) (Mean $\pm$ SD, n = 3)		
	LQC	MQC	HQC	LQC	MQC	HQC
ABZ	68.1 $\pm$ 2.7	73.7 $\pm$ 1.5	69.6 $\pm$ 0.8	94.6 $\pm$ 9.7	89 $\pm$ 1.3	97.9 $\pm$ 11.5
ABZ-OX	84.1 $\pm$ 1.8	84.3 $\pm$ 3.1	73.4 $\pm$ 1.2	94.5 $\pm$ 3.2	94.1 $\pm$ 2.5	90.5 $\pm$ 9.3
ABZ-ON	78.5 $\pm$ 5.6	85 $\pm$ 1.3	75.2 $\pm$ 1.4	89.7 $\pm$ 9.8	94.9 $\pm$ 2.1	97.1 $\pm$ 2.2
PR	76.1 $\pm$ 9.0	86.5 $\pm$ 4.4	74.6 $\pm$ 7.6	91.8 $\pm$ 1.8	97.2 $\pm$ 7.2	87.7 $\pm$ 4.2
(b)						
Analytes	Mean Extraction Recovery (%) (Mean $\pm$ SD, n = 3)			Mean Matrix Effect (%) (Mean $\pm$ SD, n = 3)		
	LQC	MQC	HQC	LQC	MQC	HQC
ABZ	48.8 $\pm$ 5.2	38.2 $\pm$ 2.3	52.2 $\pm$ 6.6	91.7 $\pm$ 4.4	100.4 $\pm$ 7.3	96.7 $\pm$ 10.8
ABZ-OX	44.9 $\pm$ 4	40.1 $\pm$ 1.6	59.4 $\pm$ 5.8	96.2 $\pm$ 5	98.2 $\pm$ 9.6	101.8 $\pm$ 8
ABZ-ON	67 $\pm$ 2.1	65.7 $\pm$ 7.2	89.5 $\pm$ 4.5	95.4 $\pm$ 2.9	101.3 $\pm$ 14.5	105.7 $\pm$ 8.3
PR	66.6 $\pm$ 4.5	52.5 $\pm$ 2.9	61.8 $\pm$ 3.6	96.8 $\pm$ 3.9	97.3 $\pm$ 1.9	99.5 $\pm$ 5.0

3.5. Calibration Curve and Extraction

The calibration curves for plasma exhibited excellent linearity across distinct concentration ranges for each analyte: 0.1–200 ng/mL for ABZ and ABZ-ON, 0.5–1000 ng/mL for ABZ-OX, and 1–2000 ng/mL for PR. The calibration curves for DBS exhibited excellent linearity across distinct concentration ranges for each analyte: 0.1–200 ng/mL for ABZ and ABZ-ON, 0.5–1000 ng/mL for ABZ-OX, and 1–2000 ng/mL for PR. The average correlation coefficient was ≥ 0.99 for both plasma and DBS and met the required acceptable limits (Figure 3). The accuracy acceptance criteria for each back-calculated concentration were set at $\pm 15\%$, except for the LLOQ, where it was $\pm 20\%$. To accept a calibration run, at least 67% of the standards, including the LLOQ and ULOQ, must meet the acceptance criterion. Different dynamic ranges were adopted for the analytes due to their distinct sensitivities, varied blood concentrations in the study samples, and differences in signal linearity. Furthermore, during the analysis, no significant peaks were detected in the blank samples following the high-quality-control (HQC) samples, and the signal observed was less than 20% of the LLOQ. This absence of a significant carry-over effect demonstrated the robustness of the analytical methodology.

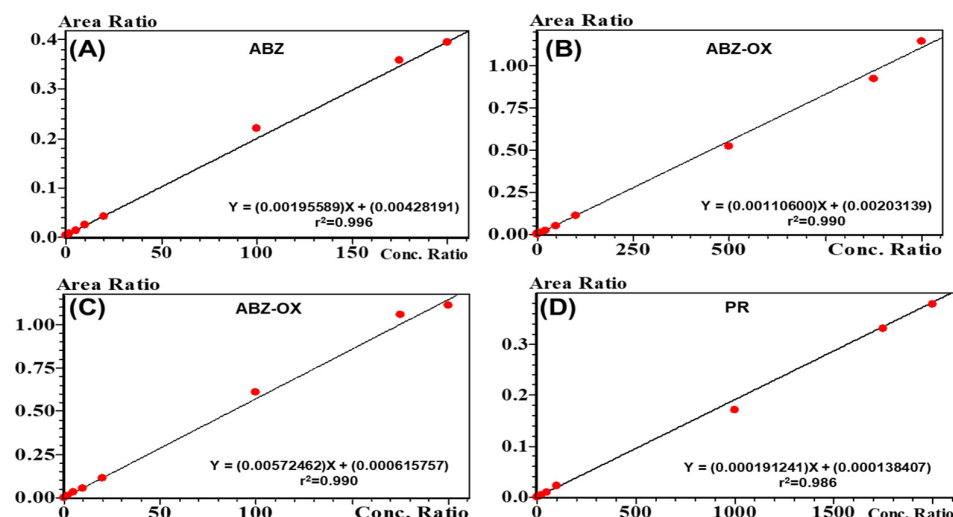


Figure 3. Calibration curve plot for (A) ABZ, (B) ABZ-OX, (C) ABZ-ON, and (D) PR, where the X-axis represents linearity concentration (ng/mL), and the Y-axis represents area ratio.

After appropriate dilution of the samples, either 2 or 5-fold, the concentration of ABZ, ABZ-OX, ABZ-ON, and PR fell within the calibration range of these compounds. The mean analyzed value of all diluted samples was within the acceptable limit range of accuracy and precision. This indicates that clinical or nonclinical samples could be diluted up to 5-fold with acceptable precision.

3.6. Stability

The stability of ABZ, ABZ-OX, ABZ-ON, and PR in plasma and DBS were assessed under various conditions enumerated in Table 4a,b. No significant degradation was observed in the analytes present in plasma samples, indicating the stability of analytes throughout the analysis. For plasma, different stability experiments were conducted, viz., long-term storage ($-80 \pm 5^\circ\text{C}$, 90 days), bench top (20°C , 8 h), autosampler (4°C , 36 h), and freeze-thaw stability ($-80 \pm 5^\circ\text{C}$). For the DBS card, the stability study was carried out, viz., bench top (20°C , 45 days), autosampler (4°C , 36 h), and long-term storage ($-80 \pm 5^\circ\text{C}$, 365 days). The stability was assayed for the three QC concentrations, which were extracted from DBS-spotted blood. No significant degradation was observed when samples were kept at the autosampler and benchtop throughout the analysis. Freeze-thaw stability was not determined for DBS spots because only fresh blood was used in the study. Furthermore, long-term storage stability was evaluated by storing the DBS samples at $-80 \pm 5^\circ\text{C}$ for 365 days, and it was found that there is no difference in terms of stability compared to freshly prepared DBS cards. In Table 4b (stability results), the MQC concentrations of stability results exceed marginally the acceptable limit of $\pm 15\%$ of the nominal concentration. However, per the FDA's guidance for bioanalytical method validation [28], calibration is said to pass when 67% of QC samples with known concentrations are within the acceptable range. Since LQC and HQC pass this criterion, the higher concentration observed for MQC samples does not impact the validation. The average percentage theoretical values of the analytes were within $\pm 15\%$ of the anticipated concentrations for the analytes at their LQC, MQC, and HQC levels for plasma and DBS. Therefore, the finding remained within acceptable limits throughout the entire validation process.

Table 4. (a) Stability results for ABZ, ABZ-OX, ABZ-ON, and PR at various storage conditions in human plasma (Mean $\pm$ SD, n = 3). (b) Stability results for ABZ, ABZ-OX, ABZ-ON, and PR at various storage conditions in human blood at DBS (Mean $\pm$ SD, n = 3).

(a)					
Analytes	QC Level	% Stability (Mean $\pm$ SD, n = 3)			
		Bench-Top (20 °C, 8 h)	Freeze-Thaw after Three Cycles (−80 $\pm$ 5 °C)	Long-Term (−80 $\pm$ 5 °C, 90 days)	Auto-Sampler (4 °C, 36 h)
ABZ	LQC	106.2 $\pm$ 0.9	108.7 $\pm$ 4.9	107.5 $\pm$ 5.5	102.1 $\pm$ 7.6
	MQC	92.4 $\pm$ 7.0	83.9 $\pm$ 2.0	91.1 $\pm$ 10.2	98.6 $\pm$ 7.5
	HQC	103.5 $\pm$ 6.7	92.6 $\pm$ 5.9	87.4 $\pm$ 5.3	101 $\pm$ 7.0
ABZ-OX	LQC	108.4 $\pm$ 6.8	111.3 $\pm$ 2.5	107.5 $\pm$ 8.8	91.3 $\pm$ 7.2
	MQC	105.1 $\pm$ 9.4	113.9 $\pm$ 4.4	114.1 $\pm$ 2	103.9 $\pm$ 4.4
	HQC	101 $\pm$ 3.6	105.8 $\pm$ 6.5	101.1 $\pm$ 0	105.8 $\pm$ 6.1
ABZ-ON	LQC	101.1 $\pm$ 13.6	111.6 $\pm$ 9.7	102.7 $\pm$ 7.5	101.6 $\pm$ 9.7
	MQC	97.6 $\pm$ 4.6	100.2 $\pm$ 13.2	114.4 $\pm$ 6.3	103.5 $\pm$ 5.7
	HQC	101.0 $\pm$ 7.0	109.6 $\pm$ 8.6	108.0 $\pm$ 2.0	98.4 $\pm$ 6.8
PR	LQC	99.3 $\pm$ 10.1	101.5 $\pm$ 10.5	112 $\pm$ 4.2	95.1 $\pm$ 9.4
	MQC	90.2 $\pm$ 10.4	100.2 $\pm$ 13.9	107.6 $\pm$ 8.1	101.2 $\pm$ 3.2
	HQC	107.4 $\pm$ 18.7	97.9 $\pm$ 4.5	102.5 $\pm$ 13.4	102.6 $\pm$ 7.5
(b)					
Analytes	QC Level	% Stability (Mean $\pm$ SD, n = 3)			Auto-Sampler (4 °C, 36 h)
		Bench-Top (20 °C, 45 Days)	Long-Term (−80 $\pm$ 5 °C, 365 Days)		
ABZ	LQC	93.2 $\pm$ 11.6	104.4 $\pm$ 7.4		93.9 $\pm$ 1.4
	MQC	95.4 $\pm$ 2.8	119.5 $\pm$ 1.5		91.1 $\pm$ 7.9
	HQC	110.3 $\pm$ 2.7	135.8 $\pm$ 0.8		106.6 $\pm$ 9.1
ABZ-OX	LQC	100.2 $\pm$ 2.9	113.5 $\pm$ 15.5		87.9 $\pm$ 15.1
	MQC	114.5 $\pm$ 14.1	86.7 $\pm$ 6.3		109.97 $\pm$ 6.4
	HQC	93.9 $\pm$ 25.5	93.0 $\pm$ 20.2		100.2 $\pm$ 6.4
ABZ-ON	LQC	83.9 $\pm$ 14.3	96.1 $\pm$ 12.7		90.6 $\pm$ 10.2
	MQC	93.2 $\pm$ 16.4	117.0 $\pm$ 2.5		84.8 $\pm$ 4.2
	HQC	96.3 $\pm$ 15.1	98.0 $\pm$ 10.5		99.6 $\pm$ 12.8
PR	LQC	99.6 $\pm$ 12.4	87.6 $\pm$ 6.		97.9 $\pm$ 12.6
	MQC	97.6 $\pm$ 7.7	108.9 $\pm$ 13.3		91.0 $\pm$ 2.5
	HQC	114.2 $\pm$ 22.2	105.2 $\pm$ 6.8		113.3 $\pm$ 14.6

4. Conclusions

The validated method was robust, reliable, and sensitive for quantifying ABZ, ABZ-OX, ABZ-ON, and PR simultaneously from the plasma and DBS, as per the US FDA guidelines and was valuable for the therapeutic drug monitoring of ABZ, ABZ-OX, ABZ-ON, and PR, which are widely utilized in the elimination of lymphatic filariasis. The validated method is efficient and suitable for high-throughput bioanalysis in any clinical setting. The accuracy, precision, selectivity, sensitivity, and stability of the data fulfilled the criteria, as per US FDA guidelines. The use of DBS for pharmacokinetic studies has

advantages due to its minimally invasive nature, reduced sample volume requirements, and improved stability of analytes during storage and transportation compared to the conventional method. Furthermore, DBS has been shown to provide reliable and accurate measurements of drug concentrations, making them a promising tool in pharmacokinetic research, and this method should be applied for therapeutic drug monitoring of ABZ, ABZ-OX, ABZ-ON, and PR in routine clinical use. Likewise, the DBS method will allow for extending these investigations in neonates and children, where the availability of blood volume for bioanalytical samples may be constrained. Compared with the reported analytical method, no one has reported the LC-MS/MS method for ABZ, ABZ-OX, ABZ-ON, and PR for both plasma and dried blood spots. We first reported a full validation method in plasma and DBS systems for the first time and compared the stability, recovery, and matrix effect, including another validation parameter. Our method differs from our previously reported method [27] in terms of the matrix and the combination of drugs used for validations. Therefore, the current LC-MS/MS method facilitates the quantification of these compounds in both plasma and DBS and offers a reliable and efficient approach for pharmacokinetic analysis in clinical and research settings.

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