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Acknowledgement of your Submission to Analytical Methods - AY-ART-12-2020-002258

1 message

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11-Dec-2020

Dear Dr Permana:

TITLE: New and sensitive HPLC-UV method for concomitant quantification of combination of antifilariasis drugs in rat plasma and organs after simultaneous oral administration

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Universitas Hasanuddin

Andi Dian Permana <andi.dian.permana@farmasi.unhas.ac.id>

Decision on submission to Analytical Methods - AY-ART-12-2020-002258

1 message

Analytical Methods <onbehalfof@manuscriptcentral.com> Mon, Dec 28, 2020 at 4:44 PM
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28-Dec-2020

Dear Dr Permana:

Manuscript ID: AY-ART-12-2020-002258
TITLE: New and sensitive HPLC-UV method for concomitant quantification of combination of antifilariasis drugs in rat plasma and organs after simultaneous oral administration

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Yours sincerely,
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Associate Editor Analytical Methods
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REVIEWER REPORT(S):
Referee: 1

Comments to the Author
Manuscript ID AY-ART-12-2020-002258
The manuscruipt reports novel methodology to differentially estimate few compounds in solution as well as in plasma or organs with HPLC. Although the method is novel and is to estimate all these compounds simultaneously with precision, it lacks some error and needs revision prior to acceptance by Analytical methods. The comments are as hereunder:
1) No internal standard is used during the analysis. It should be used to authenticate the parameters' robustness in the method. Please report the elution and 2-3 validation parameters with internal standard.
2) The extraction methodology of compounds/metabolites from organs are not clear. In which solvent did you homogenize the sample? What are the conditions?
3) Why did you choose so many gradients and elution times in Table 1? I suppose each quarter is for one analyte. Describe what are they specifically.
4) Which HPLC column did use? which HPLC also did you use? Please mention in the Materials and Methods section.
5) The discussion part is poorly written. How do you compare your methods with existing methods? explain in more details. The pharmacokinetics and concomitant tissue distribution of each of the drug or metabolite may hint at drug's metabolism and tissue protein binding together with their correlation when administered orally. You may add on discussion with this.
6) Is there any drug-drug interaction or synergism you have got? please refere to other published papers and compare your data to cast a light on this. If yes, this could interesting to optimize the doses.
7) Discuss a bit more how did you calculate t1/2 from your pharmacokinetic data. Without semilog plot or using statistical software, it could be interesting to have a light on this.
Altogether I recommend major revision of the manuscript before acceptance for publication in Analytical methods

Referee: 2

Comments to the Author
Ms. Ref. No.: AY-ART-12-2020-002258
Title: New and sensitive HPLC-UV method for concomitant quantification of combination of antifilariasis drugs in rat plasma and organs after simultaneous oral administration
Journal: Analytical Methods

REFEREE'S COMMENTS

The manuscript describes, a HLPC-UV method for simultaneous quantification of drugs (used for lymphatic filariasis treatment) such as ivermectin, albendazole, albendazole metabolites and doxycycline in plasma and tissues. The method was validated according to ICH and FDA guidelines and applied to quantify the plasma and tissue concentrations after oral administration to Wistar rats. A simple, one-step protein precipitation was used to extract both plasma and tissues. The method is described as selective, precise and accurate. The subject may result of interest to Analytical Methods. In general, the experimental design has been carried out correctly except in the aspects discussed below. Then, major revision needs to be made for a suitable presentation of the manuscript.

The following specific comments must be considered to prepare a revised version of this manuscript.

- Since diethylcarbamazine drug is used in the filariasis treatment, why was it not included in the method?

- Page 6: Different analytical portions are proposed for plasma and tissues in the calibration and in the extraction

process, how is that explained?

Line 8: “20 µL of mixed standard working solution into a 180 µL of blank rat plasma…..”
Line 29: “200 µL of drug stock solutions into a 1.8 g of blank organs to achieve the same as plasma concentrations…”
Line 42: “100 µL rat plasma samples or 100 mg of organ samples...”

- Page 6: Different injection volumes are proposed, which is the correct one?

Line 57: “10 µL was injected onto the HPLC-column…”
Line 16: “The injection volume of all samples was 50 µL”

- Page 6: Taking into account the recommended doses for these drugs and the expected concentrations, why the calibration range includes excessively high concentrations, such as 50 and 100 µg/mL? Why did the authors use that range? Especially taking into account previous works published with the same molecules (Permana et al., Journal of Pharmaceutical and Biomedical Analysis 170 (2019) 243–253). In fact, most of concentrations obtained in the present work do not reach 1 µg/mL-g, the highest being 3 µg/mL-g.

- Page 7: The meaning of this sentence is not understood, please rewrite.

Line 30: “In order to evaluate the dilution integrity, spiked plasma and organs with the analyte concentrations above the highest concentration of calibration standard solutions.

- Page 9: The authors report good low limits of quantification and selectivity in both plasma and tissues. Plasma is a simple matrix to extract, in which low limits of quantification are easily reached. However, tissues such as kidney and liver are much more complicated matrices, where interferences are frequent especially at low concentration levels for poorly selective detection methods such as UV. The authors report good selectivity, recovery, accuracy and precision in all the LLOQs without sample cleaning. The figure 2 shows the peak areas for all molecules in tissues, at high concentrations of 2.5 (IVM) and 5 µg/g (the rest of molecules). Then to arrive at the LLOQs values, these peaks areas have to be divided by 250 (2.5:0.01) or 166 (5:0.03). In this context, Could the authors guarantee the selectivity, precision, accuracy of the peaks in the LLOQs? If so, it is recommended to report the chromatograms obtained for LLOQs. If not, the LLOQs must be recalculated.

Page 7: The result and discussion section is mostly presentation of results with poor discussion that should be completed.

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Journal of Pharmaceutical Analysis

New and sensitive HPLC–UV method for concomitant quantification of antifilariasis drugs in rat plasma and organs after simultaneous oral administration

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Paper
Keywords:	Albendazole; Ivermectine; Doxycycline; HPLC-UV; Validation; Pharmacokinetic
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First Author:	Andi Dian Permana
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	Qonita Kurnia Anjani
	Emilia Utomo
	Ryan F. Donnelly
Abstract:	A combination treatment comprising ivermectin (IVM), albendazole (ABZ) and doxycycline (DOX) is often prescribed for lymphatic filariasis patients. Nevertheless, there has not been an analytical method established and documented to determine these compounds simultaneously. Herein, we reported a new high-performance liquid chromatographic method coupled with UV detector (HPLC-UV) to quantify these drugs in plasma and organs. This developed analytical method was validated according to International Conference on Harmonization (ICH) and the US Food and Drug Administration (FDA). The validated method was successfully employed to analyze IVM, ABZ along with its metabolites (albendazole sulfoxide (ABZ-OX) and albendazole sulfone (ABZ-ON)), as well as DOX in the plasma and organs of Wistar rats after simultaneous oral administration. An Xselect CSH™ C 18 HPLC column was utilized as a stationary phase, with a mobile phase consisting of 0.1% v/v trifluoracetic acid in water and acetonitrile with a run time of 20 min. The calibration curves in biological samples were found to be linear across the concentration range of 0.01–50 µg/mL for IVM, ABZ and ABZ metabolites; 0.025–100 µg/mL for DOX with an R value ≥ 0.998 in each case. The validated method was found to be selective, precise and accurate. Finally, the method developed in this study was deployed to assess the pharmacokinetic profiles and biodistribution the combination of drugs after oral administration to Wistar rats. The validated HPLC-UV method in this study provides an extensive range of prospective applications of pharmacokinetic-based studies, therapeutic drug monitoring and toxicology.
Suggested Reviewers:	Aaron Courtenay a.courtenay@ulster.ac.uk
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The Editor
Journal of Pharmaceutical Analysis

7th December 2020

Dear Sir/Madam,

I wish you to consider our manuscript entitled "**New and sensitive HPLC–UV method for concomitant quantification of antifilariasis drugs in rat plasma and organs after simultaneous oral administration"** for publication in your journal. This article describes, for the first time, the HLPC-UV method for a simultaneous quantification of plasma and organs concentration of three therapeutic agents that are widely used in treatment of lymphatic filariasis (LF), namely **ivermectin, albendazole, albendazole metabolites and doxycycline**. The method was validated according to ICH and FDA guidelines and successfully applied to quantify DOX, DEC and ABZ metabolites in the plasma and organs of Wistar rats after oral concomitant administration of the previously mentioned therapeutic drugs. In addition, a simple, one-step protein precipitation and extraction method was used to extract the four compounds efficiently. Overall, the method developed was selective, precise and accurate.

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Andi Dian Permana (on behalf of all authors)
Faculty of Pharmacy
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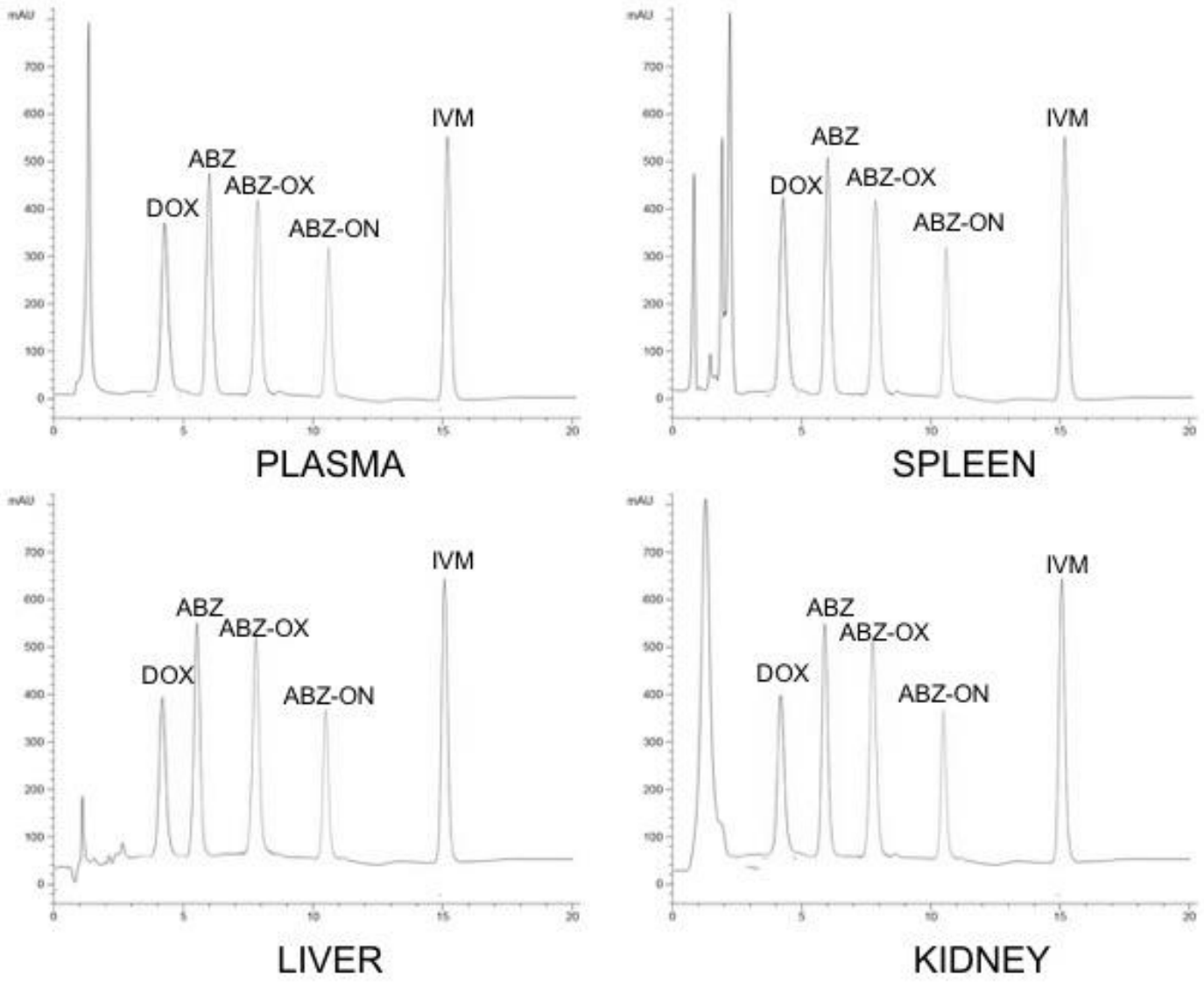
Email: andi.dian.permana@farmasi.unhas.ac.id

Highlights:

- Sensitive HPLC-UV method for quantification of antifilariasis drugs was developed.
- The method was validated according to ICH and FDA guidelines.
- The method was deployed to pharmacokinetic and biodistribution studies.

Graphical Abstract



New and sensitive HPLC–UV method for concomitant quantification of ivermectin, albendazole, albendazole metabolites and doxycycline in rat plasma and organs after simultaneous oral administration

Andi Dian Permana[1*], Elly Wahyudin[2], Ismail[3], Muh. Nur Amir[2], Muh. Raihan[3], Qonita Kurnia Anjani[4], Emilia Utomo[4], Ryan F. Donnelly[4]

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New and sensitive HPLC–UV method for concomitant quantification of antifilariasis drugs in rat plasma and organs after simultaneous oral administration

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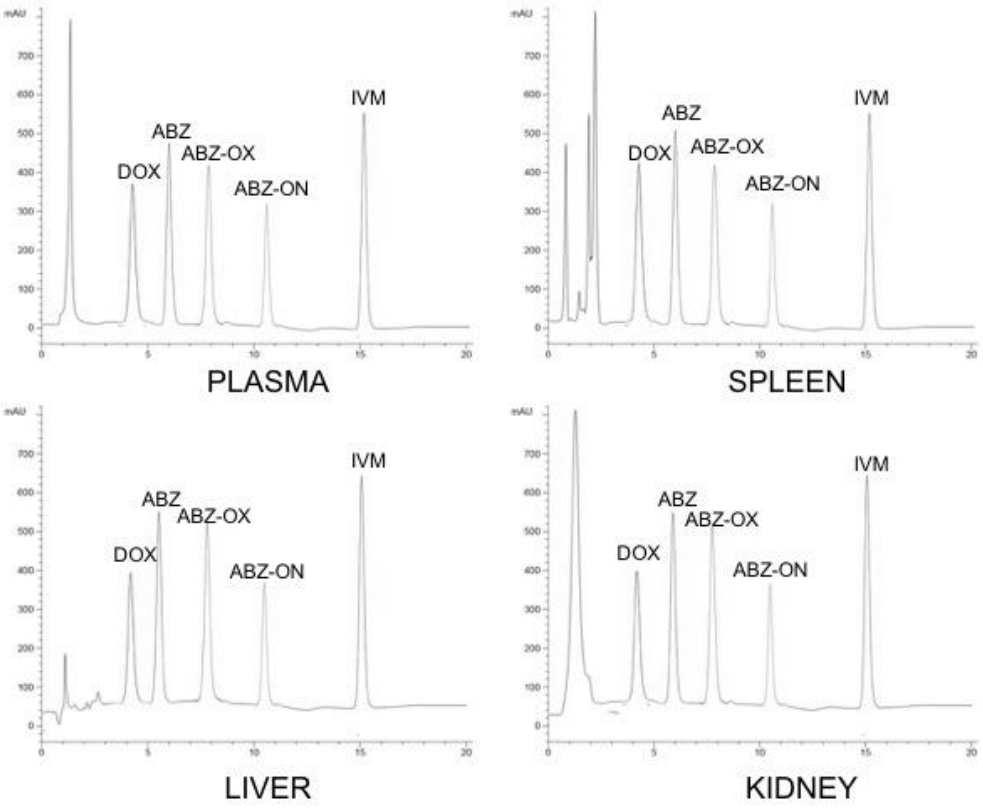
GRAPHICAL ABSTRACT


DOX
ABZ
ABZ-OX
ABZ-ON
IVM
PLASMA
SPLEEN
LIVER
KIDNEY

ABSTRACT

A combination treatment comprising ivermectin (IVM), albendazole (ABZ) and doxycycline (DOX) is often prescribed for lymphatic filariasis patients. Nevertheless, there has not been an analytical method established and documented to determine these compounds simultaneously. Herein, we reported a new high-performance liquid chromatographic method coupled with UV detector (HPLC-UV) to quantify these drugs in plasma and organs. This developed analytical method was validated according to International Conference on Harmonization (ICH) and the US Food and Drug Administration (FDA). The validated method was successfully employed to analyze IVM, ABZ along with its metabolites (albendazole sulfoxide (ABZ-OX) and albendazole sulfone (ABZ-ON)), as well as DOX in the plasma and organs of Wistar rats after simultaneous oral administration. An Xselect CSH™ C_{18} HPLC column was utilized as a stationary phase, with a mobile phase consisting of 0.1% v/v trifluoracetic acid in water and acetonitrile with a run time of 20 min. The calibration curves in biological samples were found to be linear across the concentration range of 0.01–50 µg/mL for IVM, ABZ and ABZ metabolites; 0.025–100 µg/mL for DOX with an R value $\geq$ 0.998 in each case. The validated method was found to be selective, precise and accurate. Finally, the method developed in this study was deployed to assess the pharmacokinetic profiles and biodistribution the combination of drugs after oral administration to Wistar rats. The validated HPLC-UV method in this study provides an extensive range of prospective applications of pharmacokinetic-based studies, therapeutic drug monitoring and toxicology.

Keywords: Albendazole, Ivermectine, Doxycycline, HPLC-UV, Validation, Pharmacokinetic

1. Introduction

Lymphatic filariasis (LF) is a parasitic, infectious, neglected tropical disease (NTD), and it is vector-borne, being triggered by several nematodes, mainly *Wuchereria bancrofti* or *Brugia* spp. filarial nematodes [1]. This disease is an enervating, mutilating disease, affecting impairment to the lymphatic system. Additionally, LF also results in hydrocoele and lymphoedema [2]. Initially, in 1997, LF was found to be endemic in 73 countries worldwide with more than 120 million people infected [3,4], leading to the Global Program to Eliminate Lymphatic Filariasis (GPELF) to set a program of elimination as a public health problem in 2000. LF is also a leading cause of long-term and permanent disability. It has been estimated that approximately 5.25 million disability-adjusted-life years are caused by LF. Around U$ 5.7 billion has been used annually to overcome this health issue before the establishment of the GPELF [5,6]

The GPELF elimination approach comprised two main purposes. Firstly, it was established to prevent the spread of transmission. This purpose was carried out by using the treatment of the combination of two drugs diethylcarbamazine citrate (DEC) or ivermectin (IVM) and albendazole (ABZ) to the whole population living in zones where LF is endemic, called mass drug administration (MDA). Secondly, it was launched to reduce the mobirdity of affected populations and to control morbidity. This was done by presenting basic methods, such as better-quality hygiene and skin care, particularly for infected people with lymphoedema and by performing surgery for men patient having hydrocoele [2]. With respect to the medications, specifically, IVM is an effective anti-filarial to eliminate microfilariae but not against adult filarial. Meanwhile, DEC is reported to have an ability to kill both micro and macrofilariae. Furthermore, when the combination of ABZ and either IVM or DEC is used, the treatment has been reported to improve the efficacy of MDA against LF [3]

The treatment of LF has been hampered by the fact that endosymbiotic *Wolbachia* bacteria possess a substantial responsibility in the physiology of filarial nematodes. Several studies have shown *Wolbachia* spp. infects all LF nematodes. Furthermore, it has been reported that the presence of *Wolbachia* is able to support the fertility of the nematodes by building a critical mutualistic symbiosis with the LF nematodes [7,8]. To solve this problem, although not included in MDA, it has been recommended to include doxycycline (DOX) in the treatment of LF as an antibiotic to target the bacteria endosymbiont. Therefore, by killing the bacteria, this would also decrease the viability of the adult nematodes [1]. Clinical study has revealed that in

addition to the elimination of *Wallbachia* in the LF patients, the incorporation of the use of DOX in LF therapy was able to reduce the viability of both microfilaria and adult nematodes [9,10]. Accordingly, the use of antibiotic in combination with anti-filariasis drugs is also utilized in the LF therapy.

The correlation of drug doses with response of therapeutic effect, failure of therapy, and side effects related to the combination of antibiotic and anti-filariasis is not presently discovered [11]. As a consequence, it is crucial to develop a selective and sensitive analytical method to quantify those drugs in the clinical samples. Chromatographic techniques show the prospective benefits for better reproducibility, selectivity and sensitivity in regular analysis process. Therefore, this technique is favored to analyze numerous drugs in various samples, including clinical samples. In literatures, several chromatographic techniques have been developed to analyze IVM [12–14], DEC [15,16], ABZ [17,18] and DOX [19,20] separately in different biological matrices. High performance liquid chromatography (HPLC) combined with mass spectrometric detection (MS/MS) has been widely used due to its high sensitivity [11,21]. Nevertheless, this technique involves sophisticated and high-priced instrumentation. Additionally, the samples which can be analyzed using this method are limited and some problems may occur because of ion suppression [22]. Accordingly, UV detector would be beneficial to be coupled with HPLC as it has been reported to show simplicity of instrumentation process and excellent response stability.

Chhonker et al. has reported a sensitive and selective method to clinically analyze DEC, ABZ and ABZ metabolites simultaneously in human plasma [23]. Following oral administration, ABZ is directly metabolized to its active metabolite, albendazole sulfoxide (ABZ-OX) [24]. This metabolite is further metabolized to albendazole sulfone (ABZ-ON) [25]. Therefore, in the latest study, we have also developed a novel analytical method to separate and quantify DOX, DEC, ABZ-OX and ABZ-ON in the samples obtained from rat organs and plasma [11]. However, there has been no bioanalytical method developed for simultaneous of IVM, ABZ and DOX in plasma and organ samples. Therefore, in this study, we present a new and sensitive HPLC-UV method to simultaneously quantify IVM, ABZ, ABZ metabolites and DOX in plasma as per the International Conference on Harmonization (ICH) and the US Food and Drug Administration (FDA) guidelines. The validated HPLC-UV method was then employed to assess the pharmacokinetics of the aforementioned drugs and metabolites, as well as biodistribution in body organs following simultaneous oral administration to Wistar rats.

2. Materials and methods

2.1. Chemicals and materials

Albendazole (ABZ) (purity, ≥98%), albendazole sulfone (ABZ-ON) (purity, ≥98%), doxycycline monohydrate (DOX) (purity, ≥98%) and ivermectin (IVM) (purity, ≥98%) of analytical grade were purchased from Alfa Aesar (Lancashire, UK). Albendazole sulfoxide (ABZ-OX) (purity, ≥99.9%), trifluoracetic acid of analytical grade and HPLC grade Methanol (MeOH) were purchased from Sigma–Aldrich Pte Ltd, (Singapore). HPLC column Xselect CSH™ C_{18} (Waters, 3.0 x 150 mm, 3.5 µm particle size) were purchased from Waters (Dublin, Ireland). All other chemical reagents were purchased from Sigma–Aldrich Pte Ltd, (Singapore). All other reagents were of analytical grade and purchased from standard commercial suppliers.

2.2. Preparation of stock, calibration standard and quality control samples

Stock solutions of IVM, ABZ, ABZ-OX, ABZ-ON and DOX were separately prepared by dissolving 50 mg of each drug in methanol and making up to 50 mL in a volumetric flask, obtaining a concentration of 1 mg/mL. Afterwards, the working standard solutions were prepared by diluting the stock solutions in the mobile phase. The calibration standards, and quality control (QC) solutions were prepared by serially diluting the working standard solutions obtained. A plasma calibration standard was obtained by spiking a 20 µL of mixed standard working solution into a 180 µL of blank rat plasma to achieve serial plasma concentration in the range of 0.010-50 µg/mL for IVM and 0.025-100µg/mL for ABZ, ABZ-OX, ABZ-ON and DOX, respectively. Furthermore, QC solutions in the plasma samples were prepared in three different concentrations using the same process as the calibration standards in six replicates. QC concentrations used in this study were 0.03 µg/mL (low QC), 15 µg/mL (medium QC) and 37.5 µg/mL (high QC) for IVM and 0.08 µg/mL (low QC), 25 µg/mL (medium QC) and 75 µg/mL (high QC) for ABZ, ABZ-OX, ABZ-ON and DOX.

To prepare the calibration standards of IVM, ABZ, ABZ-OX, ABZ-ON and DOX in rat organs, several organs (liver, spleen and kidney) were separately homogenized for 10 min using UltraTurrax homogenizer (IKA, model T25, impeller 10 G, Germany). The calibration standards and QC solutions were prepared by spiking a 200 µL of drug stock solutions into a 1.8 g of blank organs to achieve the same as plasma concentrations. The calibration standards

and QC solutions in organs were prepared in the same concentrations with the drug concentrations in by using ng/g as a unit. All samples were stored at −20 °C.

2.3. Sample preparation and analytes extraction

Sample preparation and analyte extraction were carried out using one-step protein precipitation with methanol. Briefly, methanol was added to 1.5 mL centrifuge tubes which contained 100 µL rat plasma samples or 100 mg of organ samples. The volume of methanol used to extract the drugs was optimized by varying the volume added, namely 100 µL, 300 µL, 500 µL, 700 µL and 900 µL. The mixtures were then vortexed for 10 min and centrifuged for 15 min at 14,000 × g. The supernatant obtained was placed into a glass vial. Subsequently, the solvent was evaporated in a fume hood for 3 h to obtain dry residue. The residue was then reconstituted in a 100 µL of mobile phase and transferred into a 0.5 mL centrifuge tube. The mixture was vortex mixed for 30 s and spun at room temperature for 15 min at 14,000 × g. The supernatant obtained was collected and a 10 µL was injected onto the HPLC-column.

2.4. Instrumentation and optimization of HPLC–UV conditions

The simultaneous analysis of all analytes was implemented utilizing a HPLC (Shimadzu Prominence, Shimadzu, Kyoto, Japan) system with LC-20AT quaternary gradient pump and Shimadzu LC solution software (*v*er. 1.21 SP1). All analyte separation was performed using Xselect CSH™ C18 column (Waters, 3.0 x 150 mm) with the particle size of 3.5 µm, fitted with a guard cartridge. The mobile phase consisted of a mixture of 0.1% v/v of trifluoracetic acid in water (mobile phase A) and methanol (MeOH) (mobile phase B). The analysis was run in the gradient condition for 20 min, as detailed in Table 1. The injection volume of all samples was 50 µL, and the column temperature was maintained at 25°C with the flow rate of 0.5 mL/min.

Table 1. Gradient conditions for HPLC-UV mobile phase.

Time	A (%)	B (%)	UV detection (nm)
0-5	75	25	270
5-12	75	25	290
12-14	30	70	245
14-18	5	95	245
18-20	75	25	270

2.5. Bioanalytical method validation

The bioanalytical method developed was then validated, as per the ICH and the US FDA guidelines [26,27]. Several parameters were validated, namely selectivity, linearity, lower limits of quantification (LLOQ), carry over, dilution integrity, accuracy, precision, extraction recovery and stability.

2.5.1. Selectivity

Selectivity of the method was evaluated by analyzing six different sources of blank plasma and organ samples from rats and corresponding samples spiked with all analytes. All preparations were carried out according to the extraction procedure.

2.5.2. Linearity, LOD and LLOQ

Linearity was assessed by preparing the calibration curves from the plasma and organ samples with working standard solution in the concentration range mentioned previously. The calibration curves were constructed at seven level concentrations on three separate times.

Lower limit of quantitation (LLOQ) and limit of detection (LOD) were calculated using the following Equations, after obtaining the standard deviation (SD) of the response and the slope of the calibration curve.

$$LOD = 3.3\sigma/S \qquad \text{(Equation 1)}$$

$$LLOQ = 10\sigma/S \qquad \text{(Equation 2)}$$

Where σ = the SD of the response of the data and S = the slope of the calibration curve.

2.5.3. Accuracy and precision

Accuracy and precision were evaluated for the LLOQ, QC samples at low, medium and high concentration, as mentioned above, in six replicates. The precision was evaluated by observing the relative standard deviation (RSD) of the responses of all solutions and the accuracy was evaluated by calculating the relative errors (RE). In this study, the intra-day and inter-day of accuracy and precision were evaluated. The RSD and RE of each replicate of all samples should be ±15 % [26,27].

2.5.4. Carry-over and dilution integrity

Carry-over evaluation was performed by initially injecting high concentration of QC samples. Afterwards, a blank solution was injected. The area of blank solution was observed, and the area should not be more than 20% of the area of a sample solution at LLOQ concentration [27].

In order to evaluate the dilution integrity, spiked plasma and organs with the analyte concentrations above the highest concentration of calibration standard solutions. In this study, all analytes spiked plasma was prepared at the concentration of 250 µg/mL for IVM and 500 µg/mL for ABZ, ABZ-OX, ABZ-ON and DOX, respectively. For organ samples, the samples were prepared in the same concentrations by using ng/g as a unit. The solutions were diluted 5 and 10 times with plasma and organs in six replicates. The accuracy and precision were finally calculated.

2.5.5. Extraction recovery

Extraction recovery of all analytes from the plasma and organ matrices were obtained by comparing the measured value of all the analytes from extracted QC samples at LLOQ, low, medium and high concentrations with the same sample concentrations prepared in the mobile phase.

2.5.6. Stability studies

Stability studies of IVM, ABZ, ABZ metabolites and DOX were carried out in rat plasma and various organ matrices under different storage conditions and treatment circumstances. The stabilities of all analyte solutions in the autosampler were evaluated for 48 h. Bench-top stability of all analytes was performed at room temperature for 24 h and long-term stability was performed at -20°C for 2 weeks. Finally, freeze-thaw stability was evaluated following freeze/thaw cycles to room temperature from -20°C storage (three cycles). The response after stability studies were compared to the initial responses of each solution.

2.6 Evaluation of the pharmacokinetics and biodistribution after oral administration of the three therapeutic agents

The animal studies were approved by the ethical Committee of the Faculty of Medicine, Hasanuddin University. Healthy male Wistar rats with an average mass of 239 ± 12 g (n=18), were acclimatized for 1 week before experimentation. The rats were administered orally 1 mL

of an aqueous suspension of 0.5% w/v carboxymethylcellulose containing three drugs which equal to a dose of 0.4 mg/kg, 15 mg/kg and 10 mg/kg of IVM, ABZ and DOX, respectively. Following oral administration of the drugs, blood samples (maximum 200 µL) were collected at pre-defined time intervals: 0.25, 0.5, 1, 2, 3, 4, 6, 24 and 48 h *via* tail vein bleeds. The blood collected was placed into a 1.5 heparinized Eppendorf tube and were centrifuged for 10 min at 4°C at 3,000 x g. The plasma samples were obtained from the supernatant and stored at -20 °C before analysis.

For biodistribution study in rat organs, following 2 and 24 h oral administration of the drugs, 6 rats were sacrificed and liver, spleen and kidney of the rats were collected. The plasma and organ samples collected were handled according to the validated method described in Section 2.3.

2.7. Statistical analysis

The data were presented as means ± standard deviation (SD). The calculation of mean, SD, %RSD and %RE were performed using Microsoft Excel® 2016 (Microsoft Corporation, Redmond, USA). For pharmacokinetic parameter calculation, PKSolver (add-in program of Microsoft Excel) was used applying non-compartmental pharmacokinetic analysis [28]. The curve of drug concentration vs time profiles was constructed. To analyze the data statistically, GraphPhad Prism® version 8.3.0 (GraphPad Software Inc., San Diego, California) was utilized, with $p < 0.05$ was indicated as a significant difference.

3. Results and Discussion

3.1. Instrumentation and optimization of HPLC–UV conditions

In this study, an RP-HPLC method with gradient method was applied. Separation of all analytes was carried out on a reverse phase C_{18} column owing to its versatility and suitability for analyzing and separating a varied range of compounds [11]. This was beneficial for the application in our study, as the compounds possessed a wide range of polarity from polar (DOX) to non-polar (IVM).

With respect to the mobile phase selection, it is important to note all compounds analyzed in this study have ionizable groups in their structures. Therefore, it is crucial to set a specific pH

value to result in desirable separation. In liquid chromatography, sometimes, broad peak becomes an issue when the mobile phase is used with the pH similar to, or close to, the pKa of the compounds analyzed. Not only that, a broad peak is usually followed by long tailing, reducing accuracy of the method [11]. Therefore, the pH of the mobile phase should be at least ± 1 pH from the pKa of the compounds analyzed. The pKa of the analytes are 12.47 for IVM, 9.60 for ABZ, 7.07 for ABZ-OX, 6.88 for ABZ-ON and 3.5 for DOX. Accordingly, 0.1% v/v of trifluoracetic acid in water with a pH of 2.5 was used in this study. Since pH 2.5 is one unit different from the pKa of DOX as the lowest pKa value, this pH was considered as a suitable selection because all compounds would be at a constant rate of ionization.

3.2. Sample preparation and analytes extraction

In this study, the sample preparation and the analyte extraction from biological matrices were performed using protein precipitation with methanol due to its speed and simplicity, as previously developed [11]. To maximize the extraction efficiency with minimum time processing, several volumes of methanol were tried. It was found that 500 µL was the optimum volume that was able to extract all compounds simultaneously. The increase of methanol volume to 700 µL and 900 µL did not show any significant improvement in extraction efficiency ($p > 0.05$) (data not shown). Accordingly, this volume was used in all sample preparation processes.

3.3 Method validation

3.3.1. Selectivity

In order to evaluate the presence of interference of endogenous substances in rat plasma and organs which can affect the separation of all analytes, a selectivity test was performed. The chromatograms of a blank plasma, liver, spleen and kidney as well as the biological matrices samples spiked with IVM, ABZ, ABZ-OX, ABZ-ON and DOX are presented in Figure 1 and Figure 2. The developed method showed that the retention time of IVM, ABZ, ABZ-OX, ABZ-ON and DOX were 15.8 min, 6.13 min, 7.98 min, 10.92 min and 4.87 min, respectively. Importantly, there was no significant interference found from endogenous substances in rat plasma and organs at the retention times of the compounds analyzed. Therefore, the method developed in this study was selective.

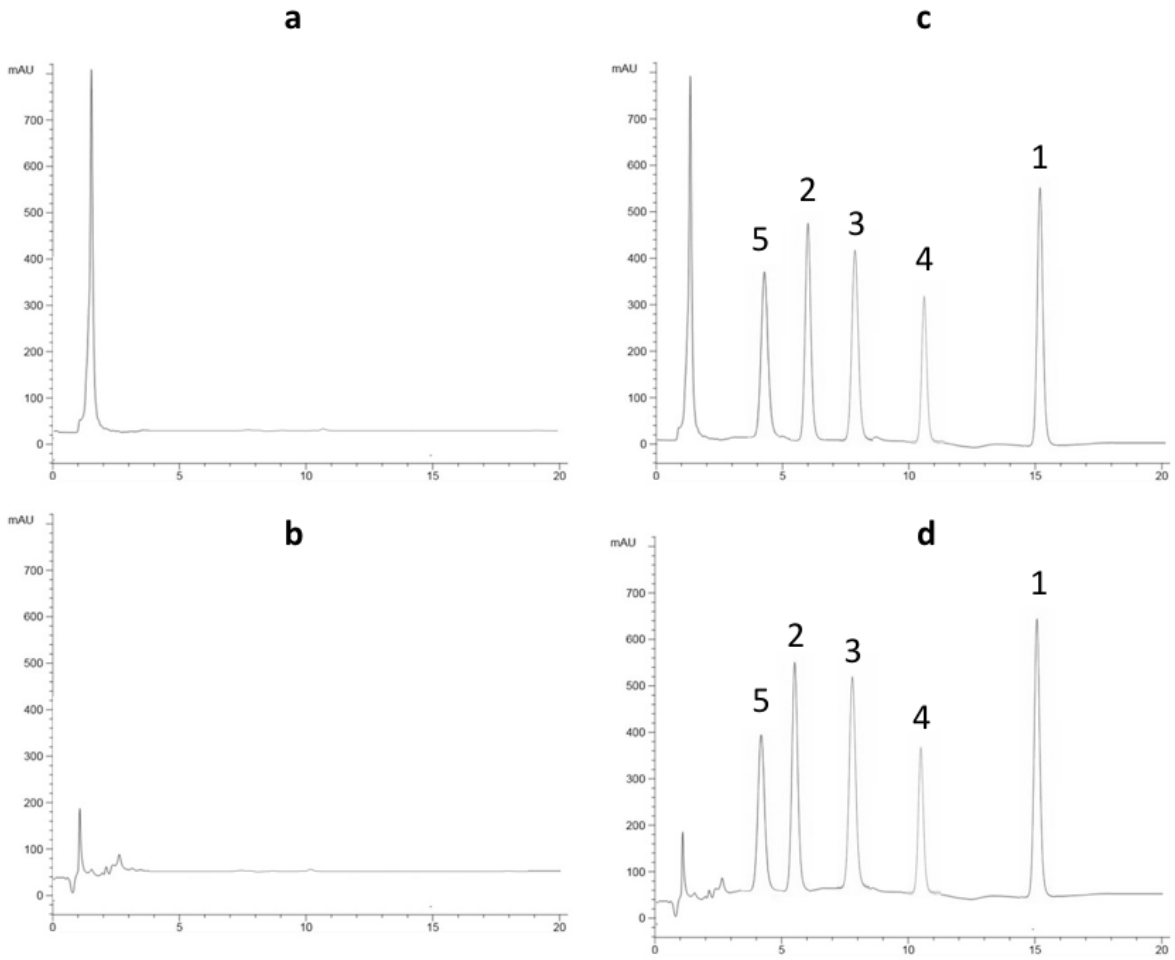


Figure 1. Representative HPLC-UV chromatograms of blank plasma (a) and blank liver (b), plasma spiked with standard solution (c) and liver spiked with standard solution (d) of IVM (2.5 μg/mL) (1), ABZ (5 μg/mL) (2), ABZ-OX (5 μg/mL) (3), ABZ-ON (5 μg/mL) (4) and DOX (5 μg/mL) (5)

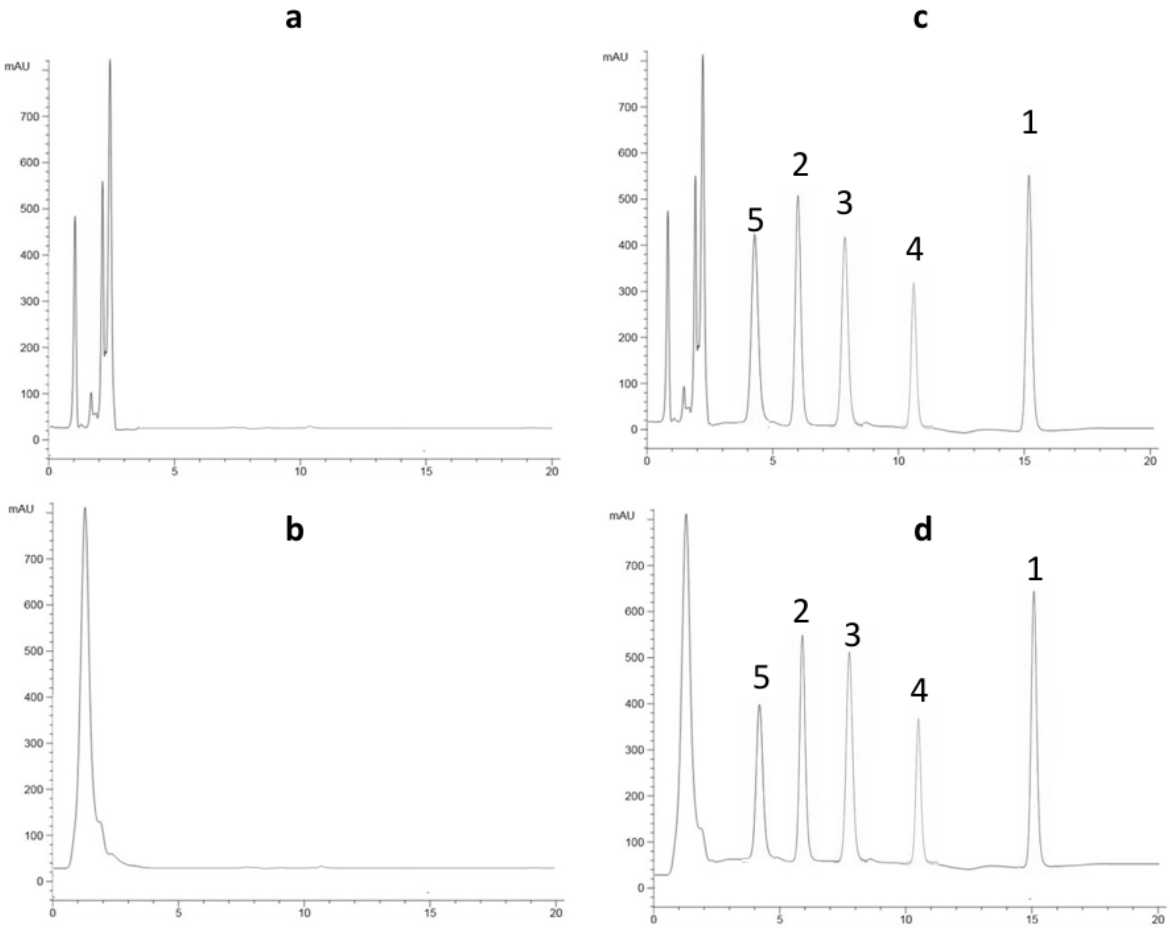


Figure 2. Representative HPLC-UV of chromatograms blank spleen (a) and blank kidney (b), spleen spiked with standard solution (c) and kidney spiked with standard solution (d) of IVM (2.5 µg/mL) (1), ABZ (5 µg/mL) (2), ABZ-OX (5 µg/mL) (3), ABZ-ON (5 µg/mL) (4) and DOX (5 µg/mL) (5)

3.3.2. Linearity, LOD and LLOQ

In order to obtain the LOD and LLOQ of this method, a set of concentration for each analyte was measured using optimized HPLC condition mentioned above. From this measurement, a calibration curve was then generated, and the equations of all compounds were used to assess the linearity and to calculate the LOD and LLOQ. The results are summarized in Table 2.

The data showed that the responses had an acceptable linear correlation within concentrations as shown by the average determination constant of $\geq$ 0.998. The range of LLOQ of all analytes, when measured in plasma, was found to be 0.013 µg/mL to 0.029 µg/mL and the LLOQ values in organs exhibited relatively similar range which is between 0.010 µg/g and 0.029 µg/g. This suggested that the method has an adequate sensitivity to examine all analytes in the plasma and organs.

Table 2. Properties of the calibration curve for quantification of all analyes with LOD and LLOQ values.

	Analyte	Slope	y-intercept	R	LOD (µg/mL)	LLOQ (µg/mL)
Plasma	IVM	1036.8	-8.43	0.999	0.006	0.01
	ABZ	409.03	-6.77	0.999	0.01	0.03
	ABZ-OX	432.54	-8.75	0.998	0.009	0.03
	ABZ-ON	414.52	-7.32	0.998	0.008	0.03
	DOX	403.76	-8.16	0.999	0.01	0.03
Liver	IVM	1102.4	-8.26	0.999	0.007	0.01
	ABZ	414.98	-6.63	0.998	0.009	0.03
	ABZ-OX	403.19	-8.93	0.998	0.009	0.03
	ABZ-ON	423.42	-7.47	0.998	0.009	0.03
	DOX	418.32	-8.32	0.998	0.01	0.03
Spleen	IVM	1021.2	-7.35	0.999	0.007	0.01
	ABZ	419.71	-5.90	0.998	0.009	0.03
	ABZ-OX	409.12	-7.94	0.999	0.01	0.03
	ABZ-ON	432.12	-7.62	0.999	0.008	0.03
	DOX	411.10	-8.49	0.998	0.009	0.03
Kidney	IVM	1114.2	-7.50	0.999	0.006	0.01
	ABZ	400.34	-6.03	0.999	0.009	0.03
	ABZ-OX	419.12	-7.79	0.998	0.009	0.03
	ABZ-ON	409.13	-7.47	0.998	0.01	0.03
	DOX	403.42	-8.32	0.999	0.01	0.03

3.3.2. Carry-over and dilution integrity

No carry-over effect was observed in the examined blank after the injection of high concentration of QC samples in the HPLC systems. This was indicated by the absence of peak that was higher than 20% of the LLOQ of the blank chromatogram. Therefore, we are confident that the interference of the analytes to a consecutive measurement was insignificant.

In order to investigate the effect of dilution on the integrity of the analyte concentration, we diluted a high concentration solution at a dilution factor of 5 and 10 times. Our finding suggested that the dilution integrity for all dilution factors were found to be satisfactory as the results exhibited values between 98.91 ± 8.65% and 102.98 ± 9.87% with precision of 9.98%-12.18%. These results indicated that the dilution integrity fell comfortably within the standard range for accuracy (between 85% and 115%) and ± 15% for precision.

3.3.3. Accuracy and Precision

The intra- and inter-day measurements of this method were accurate, since the calculated bias exhibited values within the range acceptance of ± 15% for plasma (Table 3), liver (Table 4), spleen (Table 5) and kidney (Table 6). The intra- and inter- day precisions were also reported to be acceptable. Our data showed that the %RSD in intra-day measurement were between 1.35 and 14.32 and the inter-day measurement revealed values between 2.18 and 13.26. The results of intra- and inter-day measurement are less than the standard of ± 15% bias and thus proved that the method was precise.

Table 3. Intra- and Inter-day precision and accuracy of IVM, ABZ, ABZ-OX, ABZ-ON and DOX in rat plasma (n = 6).

		Intra-day			Inter-day		
Analyte	**Concentration added (µg/mL)**	**Concentration found (µg/mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**	**Concentration found (µg/mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
IVM	0.01	0.01 ± 0.00	2.32	3.21	0.01 ± 0.00	3.25	3.65
	0.03	0.03 ± 0.00	3.12	6.67	0.03 ± 0.00	7.14	-6.67
	15	15.32 ± 0.53	3.46	2.13	14.12 ± 0.41	2.90	-5.87
	37.5	39.91 ± 1.21	3.03	6.43	35.95 ± 2.02	5.61	-4.13
ABZ	0.03	0.03 ± 0.00	4.17	6.97	0.03 ± 0.00	8.13	-7.62
	0.08	0.07 ± 0.00	2.74	-2.67	0.07 ± 0.01	8.45	-5.33
	25	23.01 ± 1.03	4.48	-7.96	27.53 ± 1.54	5.59	10.12
	75	76.08 ± 2.87	3.77	1.44	77.01 ± 3.81	4.95	2.68
ABZ-OX	0.03	0.03 ± 0.00	5.12	4.91	0.03 ± 0.00	3.15	-2.61
	0.08	0.08 ± 0.00	2.47	8.00	0.08 ± 0.01	6.10	9.33
	25	26.45 ± 1.87	7.07	5.80	27.09 ± 2.12	7.83	8.36
	75	74.21 ± 3.02	4.07	-1.05	77.25 ± 2.19	2.83	3.00
ABZ-ON	0.03	0.03 ± 0.00	5.42	4.91	0.03 ± 0.00	3.18	-4.12
	0.08	0.07 ± 0.00	1.35	-1.33	0.08 ± 0.01	4.94	8.00
	25	26.43 ± 2.01	7.60	5.72	27.41 ± 2.91	10.62	9.64
	75	76.92 ± 3.81	4.95	2.56	77.01 ± 3.33	4.32	2.68
DOX	0.03	0.03 ± 0.00	9.41	4.31	0.03 ± 0.00	2.58	-3.92
	0.08	0.08 ± 0.00	2.5	6.67	0.08 ± 0.01	5.06	5.33
	25	27.12 ± 1.15	4.24	8.48	23.11 ± 2.15	9.30	-7.56
	75	74.98 ± 3.21	4.28	-0.03	77.18 ± 4.21	5.45	2.91

Table 4. Intra- and Inter-day precision and accuracy of IVM, ABZ, ABZ-OX, ABZ-ON and DOX in rat liver (n = 6).

		Intra-day			Inter-day		
Analyte	**Concentration added (µg/mL)**	**Concentration found (µg/mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**	**Concentration found (µg/mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
IVM	0.01	0.01 ± 0.00	4.12	2.81	0.01 ± 0.00	2.78	4.13
	0.03	0.03 ± 0.00	4.09	-3.33	0.03 ± 0.00	5.03	-10.7
	15	13.98 ± 0.63	4.53	-6.80	12.98 ± 0.72	5.58	-13.5
	37.5	33.16 ± 1.32	3.97	-11.57	34.09 ± 1.66	4.88	-9.1
ABZ	0.03	0.03 ± 0.00	4.11	3.92	0.03 ± 0.00	3.73	8.12
	0.08	0.07 ± 0.01	3.59	-2.67	0.07 ± 0.00	4.41	-10.1
	25	23.91 ± 1.40	5.87	-4.36	25.75 ± 1.86	7.22	3
	75	73.12 ± 3.61	4.94	-2.51	81.90 ± 4.98	6.07	9.2
ABZ-OX	0.03	0.03 ± 0.00	6.15	3.21	0.03 ± 0.00	4.32	5.32
	0.08	0.08 ± 0.01	3.24	9.33	0.07 ± 0.01	3.98	-10.7
	25	27.09 ± 2.51	9.26	8.36	22.95 ± 2.61	11.39	-8.2
	75	72.98 ± 3.89	5.33	-2.69	84.75 ± 5.56	6.56	13
ABZ-ON	0.03	0.03 ± 0.00	3.21	3.41	0.03 ± 0.00	4.11	-3.41
	0.08	0.07 ± 0.00	1.77	-8.00	0.07 ± 0.00	2.18	-9.8
	25	27.67 ± 2.76	9.96	10.68	27.45 ± 3.36	12.25	9.8
	75	72.72 ± 4.72	6.48	-3.04	67.43 ± 5.38	7.98	-10.1
DOX	0.03	0.03 ± 0.00	8.11	3.18	0.03 ± 0.00	3.12	6.17
	0.08	0.07 ± 0.00	3.28	-2.67	0.07 ± 0.00	4.03	-1.8
	25	26.19 ± 1.46	5.55	4.76	22.80 ± 1.56	6.83	-8.8
	75	73.21 ± 4.11	5.61	-2.39	81.90 ± 5.65	6.90	9.2

400 **Table 5.** Intra- and Inter-day precision and accuracy of IVM, ABZ, ABZ-OX, ABZ-ON and DOX in rat spleen (n = 6).

		Intra-day			Inter-day		
Analyte	**Concentration added (µg/mL)**	**Concentration found (µg/mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**	**Concentration found (µg/mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
IVM	0.01	0.01 ± 0.00	8.10	2.82	0.01 ± 0.00	1.89	3.83
	0.03	0.03 ± 0.00	6.74	1.50	0.03 ± 0.00	5.85	-14.63
	15	14.68 ± 1.09	7.48	-2.14	13.21 ± 1.66	12.53	-11.93
	37.5	34.82 ± 2.28	6.55	-7.15	32.43 ± 1.84	5.68	-13.52
ABZ	0.03	0.03 ± 0.00	4.17	-5.15	0.03 ± 0.00	4.17	-5.19
	0.08	0.08 ± 0.01	5.92	2.20	0.06 ± 0.00	5.14	-14.67
	25	25.11 ± 2.43	9.68	0.42	24.05 ± 2.02	8.40	-3.80
	75	76.78 ± 6.26	8.15	2.37	76.49 ± 5.41	7.07	1.99
ABZ-OX	0.03	0.03 ± 0.00	7.16	4.02	0.03 ± 0.00	-8.23	9.13
	0.08	0.09 ± 0.01	5.34	14.80	0.07 ± 0.00	4.63	-8.00
	25	28.44 ± 4.07	14.32	13.78	21.44 ±2.84	13.26	-14.26
	75	76.63 ± 6.74	8.80	2.17	79.16 ± 6.04	7.63	5.54
ABZ-ON	0.03	0.03 ± 0.00	4.19	-4.16	0.03 ± 0.00	4.19	-7.73
	0.08	0.07 ± 0.00	2.92	-3.40	0.07 ± 0.01	2.53	-13.33
	25	28.05 ± 3.18	11.33	12.20	25.64 ± 2.16	8.43	2.55
	75	76.36 ± 10.70	10.70	1.81	68.64 ± 6.37	9.28	-8.48
DOX	0.03	0.03 ± 0.00	8.18	-4.75	0.03 ± 0.00	5.19	-8.19
	0.08	0.08 ± 0.01	5.40	2.20	0.07 ± 0.00	4.69	-8.28
	25	27.50 ± 2.52	9.16	10.00	21.30 ± 1.69	7.95	-14.82
	75	76.87 ± 7.11	9.25	2.49	76.49 ± 6.14	8.03	1.99

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Table 6. Intra- and Inter-day precision and accuracy of IVM, ABZ, ABZ-OX, ABZ-ON and DOX in rat kidney (n = 6).

		Intra-day			Inter-day		
Analyte	**Concentration added (µg/mL)**	**Concentration found (µg/mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**	**Concentration found (µg/mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
IVM	0.01	0.01 ± 0.00	3.42	-1.81	0.01 ± 0.00	4.69	-3.13
	0.03	0.03 ± 0.00	6.83	3.29	0.03 ± 0.00	7.40	13.44
	15	15.98 ± 1.04	6.51	6.56	16.32 ± 0.87	5.32	8.80
	37.5	39.24 ± 1.56	3.98	4.64	35.42 ± 2.54	7.18	-5.55
ABZ	0.03	0.03 ± 0.00	3.19	8.28	0.03 ± 0.00	7.13	8.21
	0.08	0.08 ± 0.00	5.15	3.25	0.09 ± 0.01	6.50	13.39
	25	27.43 ± 2.31	8.42	9.72	23.32 ± 2.15	9.21	-6.72
	75	80.98 ± 5.74	7.09	7.97	78.43± 7.01	8.94	4.57
ABZ-OX	0.03	0.03 ± 0.00	6.19	-3.11	0.03 ± 0.00	3.19	-4.01
	0.08	0.08 ± 0.01	4.64	11.32	0.08 ± 0.01	5.86	8.00
	25	25.94 ± 3.23	12.46	3.75	28.48 ± 4.77	16.76	13.93
	75	73.21 ± 5.60	7.65	-2.39	80.40 ± 7.76	9.65	7.20
ABZ-ON	0.03	0.03 ± 0.00	9.13	-4.11	0.03 ± 0.00	5.11	6.11
	0.08	0.08 ± 0.01	2.54	4.87	0.08 ± 0.01	3.20	10.67
	25	27.87 ± 3.98	14.29	11.48	21.36 ± 2.28	10.66	-14.56
	75	83.05 ± 7.73	9.31	10.74	78.98 ± 9.27	11.73	5.31
DOX	0.03	0.03 ± 0.00	8.15	6.15	0.03 ± 0.00	5.43	-7.72
	0.08	0.08 ± 0.01	4.70	10.98	0.07 ± 0.01	5.93	-9.33
	25	25.77 ± 2.06	7.97	3.07	28.30 ± 3.74	13.21	13.19
	75	81.43 ± 6.55	8.05	8.57	79.09 ± 8.03	10.15	5.45

3.3.4. Extraction recoveries

The recovery of analytes upon extraction was obtained by comparing the concentrations of spiked QC samples at three different concentrations to the concentration resulted from the measurements of these samples. Table 7-10 shows the results. The absolute mean recoveries for all control samples were between 65.98 ± 8.05 and 89.15 ± 9.27, between 83.21 ± 6.54 and 99.61 ± 8.46, between 83.41 ± 11.02 and 98.55 ± 13.76, between 79.43 ± 10.24 and 98.68 ± 4.57, between 96.12 ± 7.41 and 101.60 ± 9.71 for IVM, ABZ, ABZ-OX, ABZ-ON and DOX respectively. Although the yield of IVM in this method was relatively low in comparison with the recoveries of DOX, ABZ, ABZ-OX and ABZ-ON, the result was consistent with the data previously reported by a study in which the average recovery was approximately 62.8% [29]. In addition, the %RSD of all these samples were also calculated and it was found to be within ± 15% across all concentrations. Therefore, the method was considered to be consistent, precise and reproducible.

Table 7. Mean extraction recoveries of IVM, ABZ, ABZ-OX, ABZ-ON and DOX in rat plasma (n=6)

Analyte	Concentration added (µg/mL)	% Extraction Recovery ± SD	% RSD
IVM	0.01	67.93 ± 5.98	8.80
	0.03	69.01 ± 6.09	8.82
	15	65.98 ± 8.05	12.20
	37.5	74.91 ± 8.87	11.84
ABZ	0.03	93.21 ± 8.32	8.93
	0.08	87.76 ± 7.19	8.19
	25	95.19 ± 6.53	6.86
	75	93.87 ± 8.13	8.67
ABZ-OX	0.03	85.33 ± 7.54	8.84
	0.08	87.98 ± 8.43	9.58
	25	83.42 ± 8.34	9.99
	75	91.17 ± 9.52	10.44
ABZ-ON	0.03	87.98 ± 5.84	6.64
	0.08	89.92 ± 9.98	11.09
	25	83.19 ± 7.21	9.07
	75	79.45 ± 8.45	10.63
DOX	0.03	97.21 ± 9.98	10.27
	0.08	96.12 ± 7.41	7.71
	25	100.32 ± 10.43	10.39
	75	98.23 ± 8.98	9.14

Table 8. Mean extraction recoveries of IVM, ABZ, ABZ-OX, ABZ-ON and DOX in rat liver (n=6)

Analyte	Concentration added (µg/mL)	% Extraction Recovery ± SD	% RSD
IVM	0.01	70.98 ± 5.86	8.26
	0.03	71.03 ± 6.36	8.95
	15	67.93 ± 4.37	6.44
	37.5	73.23 ± 6.36	8.69
ABZ	0.03	90.54 ± 8.54	9.43
	0.08	83.21 ± 6.54	7.86
	25	93.24 ± 10.39	11.14
	75	91.98 ± 9.95	10.81
ABZ-OX	0.03	90.21 ± 8.92	9.89
	0.08	88.77 ± 6.29	7.09
	25	83.41 ± 11.02	13.21
	75	92.54 ± 10.80	11.67
ABZ-ON	0.03	89.54 ± 8.14	9.09
	0.08	91.32 ± 3.54	3.87
	25	79.43 ± 10.24	12.89
	75	81.09 ± 11.51	14.20
DOX	0.03	99.21 ± 8.32	8.39
	0.08	98.32 ± 7.05	7.17
	25	99.32 ± 13.00	13.09
	75	101.09 ± 12.41	12.28

Table 9. Mean extraction recoveries of IVM, ABZ, ABZ-OX, ABZ-ON and DOX in rat spleen (n=6)

Analyte	Concentration added (µg/mL)	% Extraction Recovery ± SD	% RSD
IVM	0.01	80.43 ± 7.64	9.49
	0.03	73.30 ± 8.86	12.09
	15	74.72 ±5.83	7.80
	37.5	82.02 ± 3.91	4.77
ABZ	0.03	93.21 ± 5.43	5.83
	0.08	90.70 ± 5.60	6.17
	25	96.22 ± 9.71	10.09
	75	99.61 ± 8.46	8.49
ABZ-OX	0.03	93.18 ± 8.65	9.28
	0.08	91.61 ± 5.10	5.57
	25	85.16 ± 12.71	14.93
	75	96.52 ± 8.85	9.17
ABZ-ON	0.03	93.19 ± 9.54	10.23
	0.08	94.61 ± 2.88	3.04
	25	81.97 ± 6.54	7.98
	75	83.60 ± 9.32	11.15
DOX	0.03	100.21 ± 9.54	9.52
	0.08	100.68 ± 5.67	5.63
	25	101.60 ± 9.71	9.55
	75	99.07 ± 9.55	9.64

Table 10. Mean extraction recoveries of IVM, ABZ, ABZ-OX, ABZ-ON and DOX in rat kidney (n=6)

Analyte	Concentration added (µg/mL)	% Extraction Recovery ± SD	% RSD
IVM	0.01	85.17 ± 8.14	9.56
	0.03	76.45 ± 8.18	10.70
	15	76.29 ± 9.05	11.87
	37.5	89.15 ± 9.27	10.39
ABZ	0.03	97.01 ± 8.32	8.58
	0.08	96.23 ± 9.04	9.40
	25	97.47 ± 12.18	12.49
	75	97.62 ± 12.62	12.93
ABZ-OX	0.03	94.76 ± 8.32	8.78
	0.08	89.78 ± 7.61	8.47
	25	88.82 ± 7.37	8.30
	75	98.55 ± 13.76	13.96
ABZ-ON	0.03	93.43 ± 9.02	9.65
	0.08	98.68 ± 4.57	4.63
	25	82.96 ± 8.42	8.43
	75	86.28 ± 10.53	12.21
DOX	0.03	93.18 ± 8.34	8.95
	0.08	98.23 ± 8.42	8.58
	25	99.01 ± 14.40	14.54
	75	97.09 ± 14.25	14.68

3.3.5. Stability

As shown in Table 11, the average recoveries were found to be between 95.89 ± 9.27 and 101 ± 8.54%. Importantly, the % RSD values were in the acceptance range of ± 15%. Therefore, lll samples were found to be stable in all tested conditions. Although our finding suggested an excellent stability of all analytes in matrices examined in this experiment, it may be important to note that IVM in blood samples were reported to be unstable after 2-3 month of storage and when it was tested in a repetitive freezing and thawing condition [30].

Table 11. Mean stability recoveries of IVM, ABZ, ABZ-OX, ABZ-ON and DOX at different storage conditions (n=3)

		% Stability recoveries (mean ± SD)			
	Analyte	**Autosampler (48 h)**	**Bench-top (24 h)**	**Long-term (2 weeks)**	**Freeze-thaw (3 cycles)**
Plasma	IVM	101.03 ± 8.54	98.18 ± 4.65	100.93 ± 9.87	98.92 ± 6.43
	ABZ	99.43 ± 7.98	97.79 ± 8.27	101.33 ± 8.62	96.27 ± 8.33
	ABZ-OX	97.98 ± 6.87	98.91 ± 7.98	98.21 ± 8.76	98.21 ± 8.43
	ABZ-ON	99.87 ± 9.32	98.31 ± 9.98	99.28 ± 8.82	97.32 ± 9.01
	DOX	98.43 ± 9.11	97.81 ± 2.55	98.89 ± 7.65	97.98 ± 2.49
Liver	IVM	99.98 ± 3.43	98.87 ± 7.32	98.38 ± 7.43	98.91 ± 9.03
	ABZ	97.81 ± 8.32	95.89 ± 9.27	99.29 ± 7.32	98.13 ± 4.32
	ABZ-OX	97.18 ± 8.87	97.98 ± 8.98	97.91 ± 7.09	99.28 ± 2.09
	ABZ-ON	98.81 ± 4.52	99.98 ± 4.32	99.21 ± 4.59	98.81 ± 5.64
	DOX	100.98 ± 8.12	100.32 ± 5.43	100.31 ± 9.73	99.08 ± 8.45
Spleen	IVM	98.76 ± 8.19	98.92 ± 9.87	99.29 ± 9.04	96.53 ± 4.94
	ABZ	97.28 ± 8.23	97.21 ± 5.43	97.23 ± 3.43	97.43 ± 9.03
	ABZ-OX	99.02 ± 6.98	98.91 ± 8.98	96.98 ± 8.43	98.81 ± 9.93
	ABZ-ON	100.19 ± 10.01	96.45 ± 6.76	97.39 ± 2.98	98.98 ± 5.43
	DOX	98.81 ± 3.12	99.23 ± 3.91	98.39 ± 8.98	97.87 ± 3.21
Kidney	IVM	97.16 ± 7.62	98.87 ± 7.54	98.93 ± 2.65	98.13 ± 3.98
	ABZ	96.87 ± 8.09	99.89 ± 9.32	99.09 ± 8.73	97.09 ± 4.08
	ABZ-OX	99.17 ± 2.39	97.65 ± 3.54	100.32 ± 10.21	98.07 ± 3.89
	ABZ-ON	100.87 ± 10.01	98.13 ± 3.98	98.82 ± 4.39	96.92 ± 3.54
	DOX	98.64 ± 8.83	98.91 ± 7.65	99.91 ± 8.65	97.98 ± 6.49

3.5. *Application of the method to pharmacokinetic and biodistribution study of IVM, ABZ and DOX*

The LLOQ values obtained using this method were considerably higher than in previous studies performed using LC MS/MS [23,29,30]. However, the method developed in this study was successfully applied to investigate the pharmacokinetic profiles and biodistribution of simultaneous *in vivo* administration of IVM, ABZ and DOX. Figure 3 gives an overview of pharmacokinetic profiles of the all substances, while the pharmacokinetic parameters are shown in Table 12.

The maximum plasma concentration (C_{max}) of the IVM were found to be 0.026 ± 0.01 µg/mL. The C_{max} of this drug was obtained after 24 h of administration. Additionally, the elimination half-life ($T_{1/2}$) of IVM was found to be 10.72 ± 2.58 h. The C_{max} of ABZ were 0.16 ± 0.03 µg/mL and was achieved after 0.25 h of administration. The C_{max} of its metabolites, ABZ-OX and ABZ-ON, were detected at concentrations of 1.32 ± 0.32 and 0.098 ± 0.023 µg/mL respectively. The time in which the C_{max} of ABZ-OX and ABZ-ON was obtained (T_{max}) were 3 and 2 h, respectively. The C_{max} of DOX was achieved after 2 h of administration, particularly at a concentration of 3.05 ± 0.79 µg/mL. A previous study reported T_{max} values for ABZ-OX, ABZ-ON and DOX that were consistent with our findings, even though the study obtained the T_{max} of these metabolites by administering ABZ-OX orally [11].

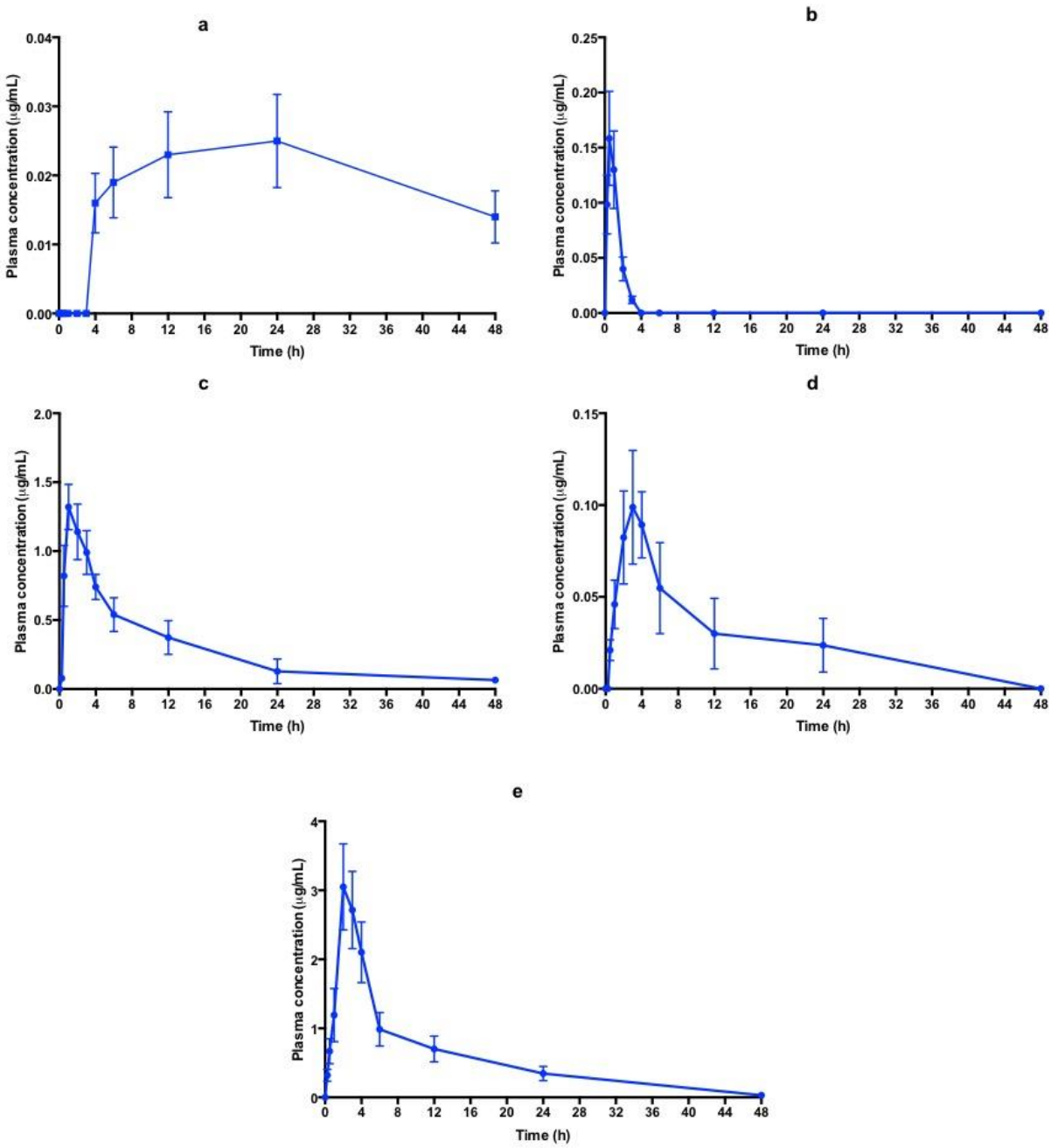

Figure 3. The mean plasma concentration and time profile of IVM (a), ABZ (b), ABZ-OX (c), ABZ-ON (d) and DOX (e) after oral administration (means ± SD, n = 6 for each group).

Table 12. Pharmacokinetics parameters of IVM, ABZ, ABZ-OX, ABZ-ON and DOX after oral administration in Wistar rats (means ± SD, n = 6 for each group).

Parameters	IVM	ABZ	ABZ-OX	ABZ-ON	DOX
Dose (mg/kg)	0.4	15	-	-	10
C_{max} (µg/mL)	0.026 ± 0.01	0.16 ± 0.03	1.32 ± 0.32	0.10 ± 0.02	3.05 ± 0.79
T_{max} (h)	24	0.5	1	3	2
AUC_{0-t} (µg/mLh)	1.08 ± 0.23	0.24 ± 0.05	13.16 ± 3.12	0.99 ± 0.23	26.99 ± 5.67
AUC_{0-INF} (µg/mLh)	1.08 ± 0.19	0.24 ± 0.05	14.35 ± 2.76	1.34 ± 0.28	27.35 ± 5.98
$T_{1/2}$ (h)	10.72 ± 2.58	2.99 ± 0.71	12.69 ± 2.61	10.38 ± 2.98	8.24 ± 1.98

This method was also applicable to measure the concentration of all drugs in rat organs such as liver, spleen and kidney specifically at 2 and 24 h after oral administration. Figure 4 summarizes the exposure of all drugs and the metabolites of ABZ in the examined organs. IVM in particular, was not detected in all organs at 2 h but found in the three organs after 24 h. On the other hand, ABZ was detected in the liver at a relatively higher concentration at 2 h after delivery and decreased to 0.03 ± 0.01 µg/mL 24 h later, while in the kidney and the spleen, it was not measurable after 24 h. In 2 h after administration, ABZ-OX was found in the liver, the spleen and the kidney at a concentration of 0.87 ± 0.22 µg/mL, 0.12 ± 0.03 µg/mL and 0.21 ± 0.04 µg/mL respectively. However, after 24 h, ABZ-OX was not present in spleen and kidney, while 0.027 ± 0.006 µg/mL was still detected in the liver. ABZ-ON was also found in the liver, the spleen and the kidney 2 h after administration at a concentration of 0.14 ± 0.04 µg/mL, 0.03 ± 0.01 µg/mL, and 0.07 ± 0.02 µg/mL, respectively, but this metabolite was not present in the spleen and the kidney 24 h later. We also found that DOX was distributed in all organs at a relatively higher concentration than the other analytes. Therefore, we described the order of the distribution as follow: liver > kidney > spleen.

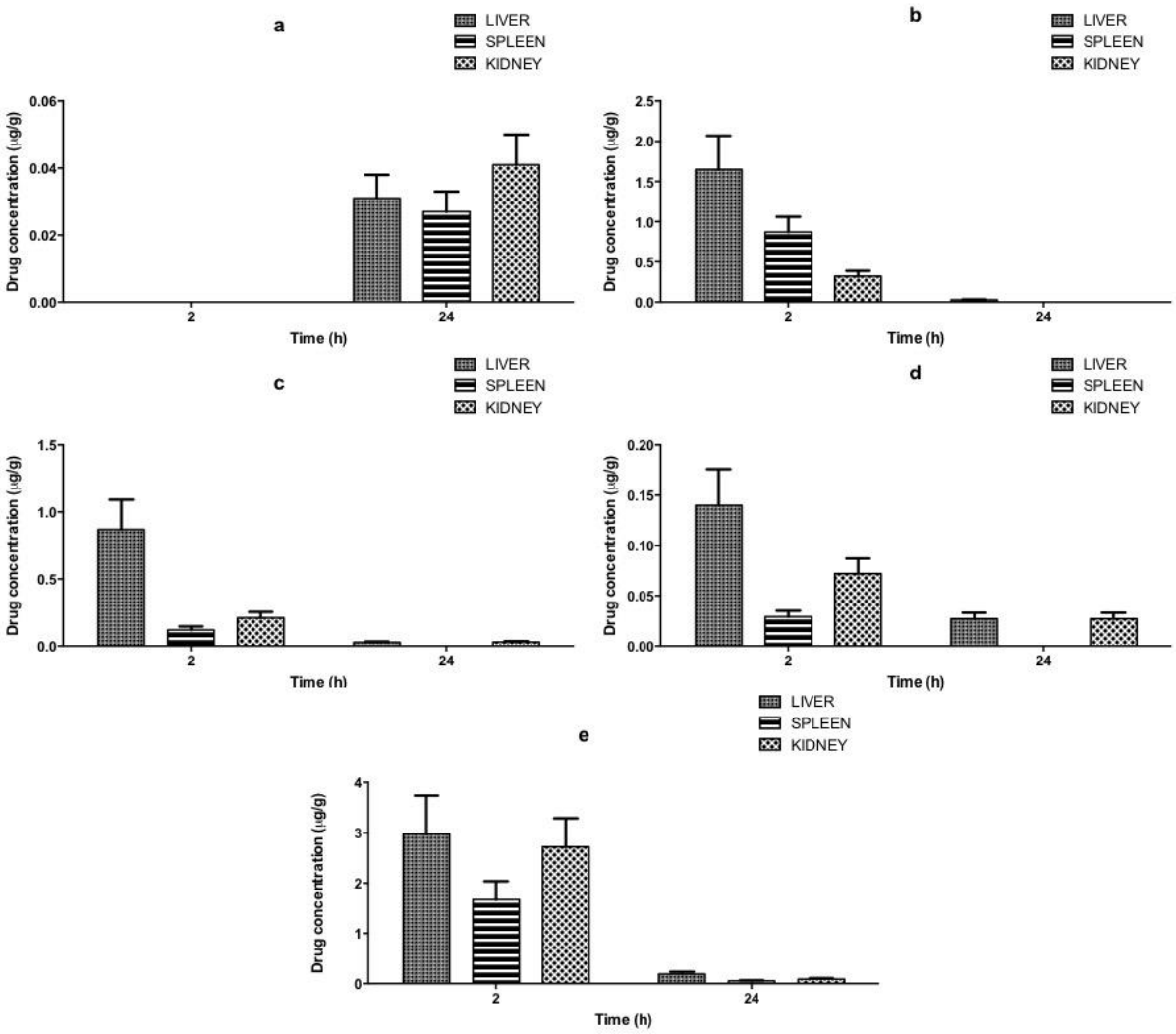

Figure 4. Biodistribution profile of IVM (a), ABZ (b), ABZ-OX (c), ABZ-ON (d) and DOX (e) in the liver, the spleen and the kidney after oral administration (n = 6 for each group).

4. Conclusion

This study was designed to develop and validate a HPLC-UV method for a simultaneous analysis of the drugs used in the lymphatic filariasis treatment, namely IVM, ABZ and its metabolites (ABZ-OX and ABZ-ON), as well as DOX. A bioanalytical method was developed in plasma and different organs, namely liver, spleen and kidney. The developed method was successfully validated as per ICH and FDA bioanalytical guidelines. The validated method was found to be linear, accurate, and precise over a reasonable concentration. The method gave LLOQ values of between 0.01 to 0.03 µg/mL in plasma and between 0.01 and 0.03 µg/g in organs. The method was selective with consistent recovery, and no intervention was found from the endogenous compounds of the matrices for all analytes. In conclusion, the novel validated method enabled the simultaneous quantification of IVM, ABZ, ABZ-OX, ABZ-ON

and DOX in the pharmacokinetic and biodistribution studies of for the first time in Wistar rats. Accordingly, this bioanalytical method offers a wide range of the quantification of anti-filariasis drugs in future pharmacokinetic and biodistribution studies, as well as the applications of therapeutic drug monitoring.

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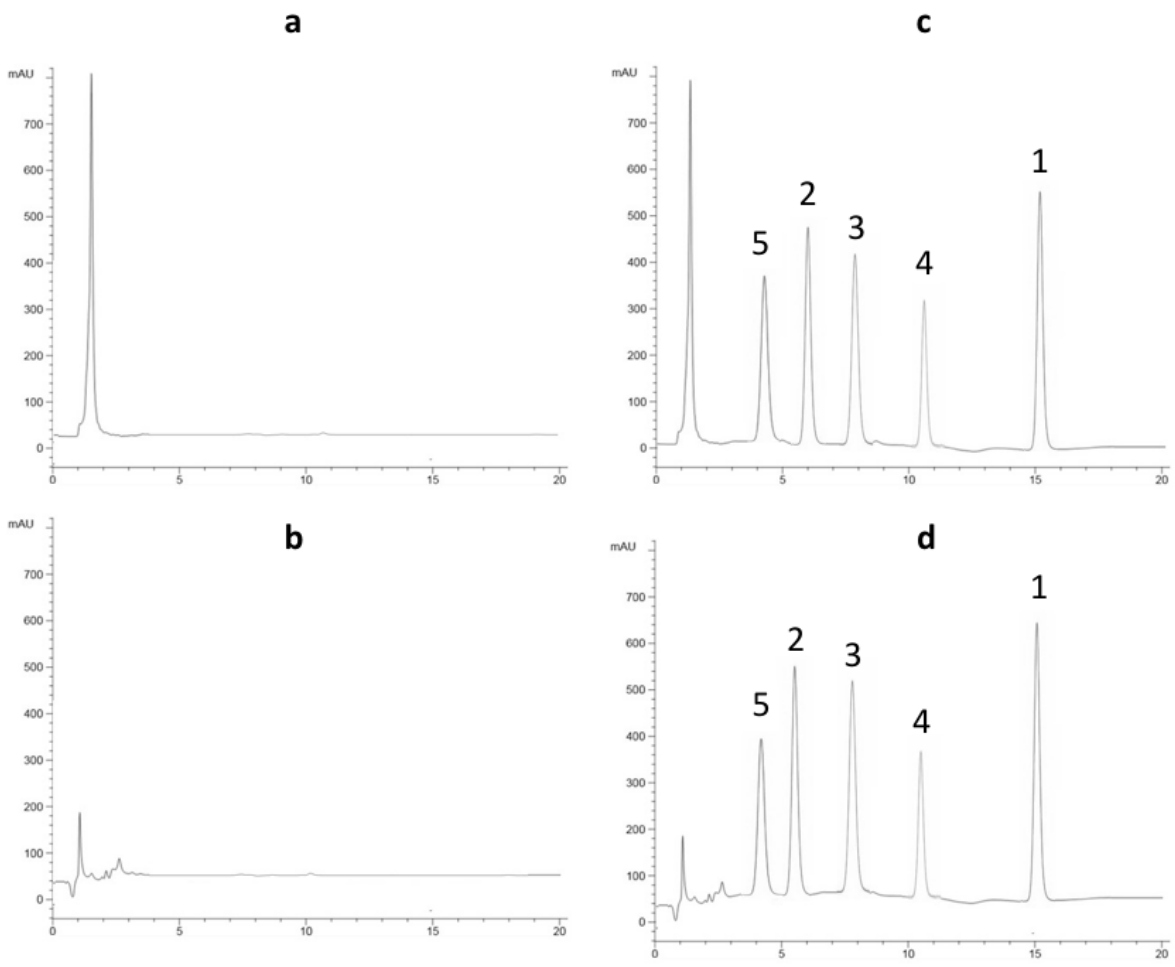

Figure 1. Representative HPLC-UV chromatograms of blank plasma (a) and blank liver (b), plasma spiked with standard solution (c) and liver spiked with standard solution (d) of IVM (2.5 µg/mL) (1), ABZ (5 µg/mL) (2), ABZ-OX (5 µg/mL) (3), ABZ-ON (5 µg/mL) (4) and DOX (5 µg/mL) (5)

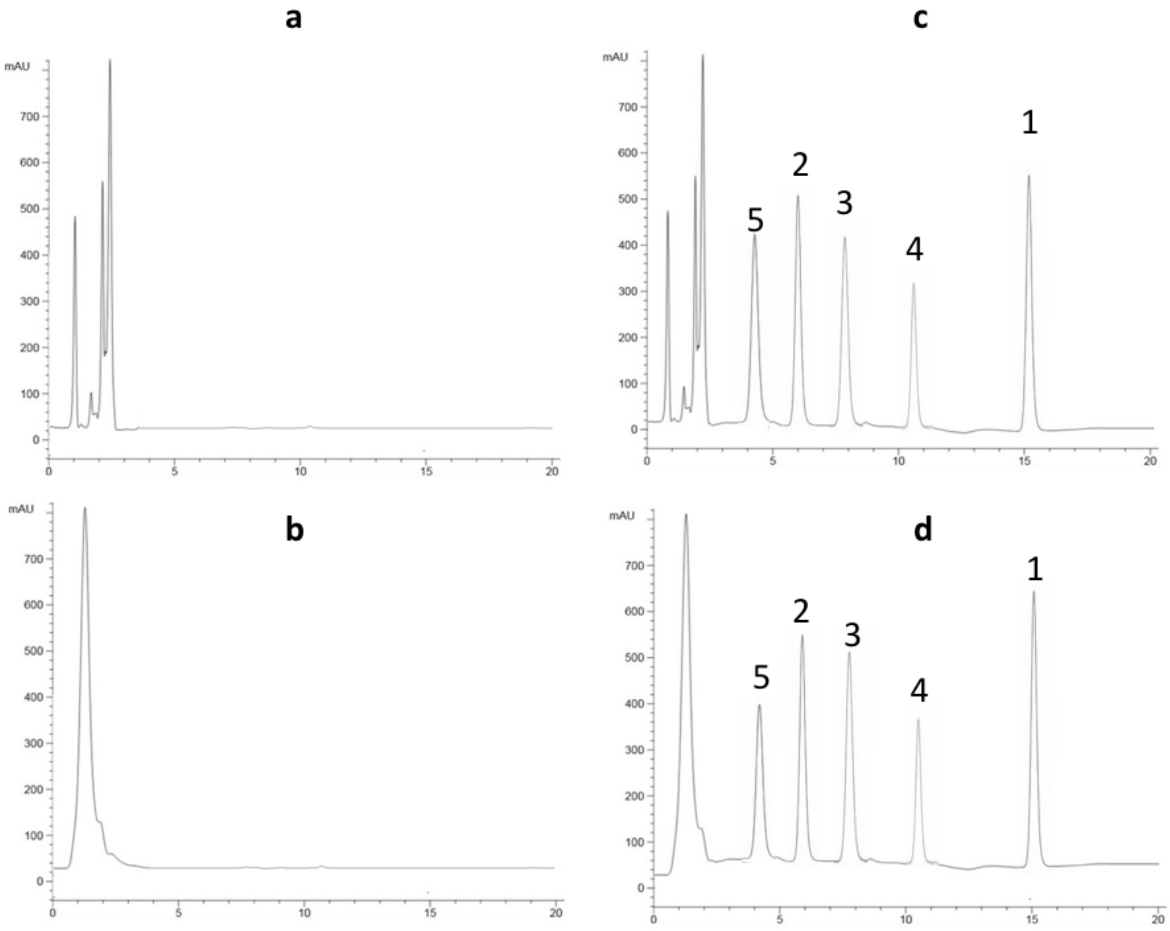

Figure 2. Representative HPLC-UV of chromatograms blank spleen (a) and blank kidney (b), spleen spiked with standard solution (c) and kidney spiked with standard solution (d) of IVM (2.5 µg/mL) (1), ABZ (5 µg/mL) (2), ABZ-OX (5 µg/mL) (3), ABZ-ON (5 µg/mL) (4) and DOX (5 µg/mL) (5)

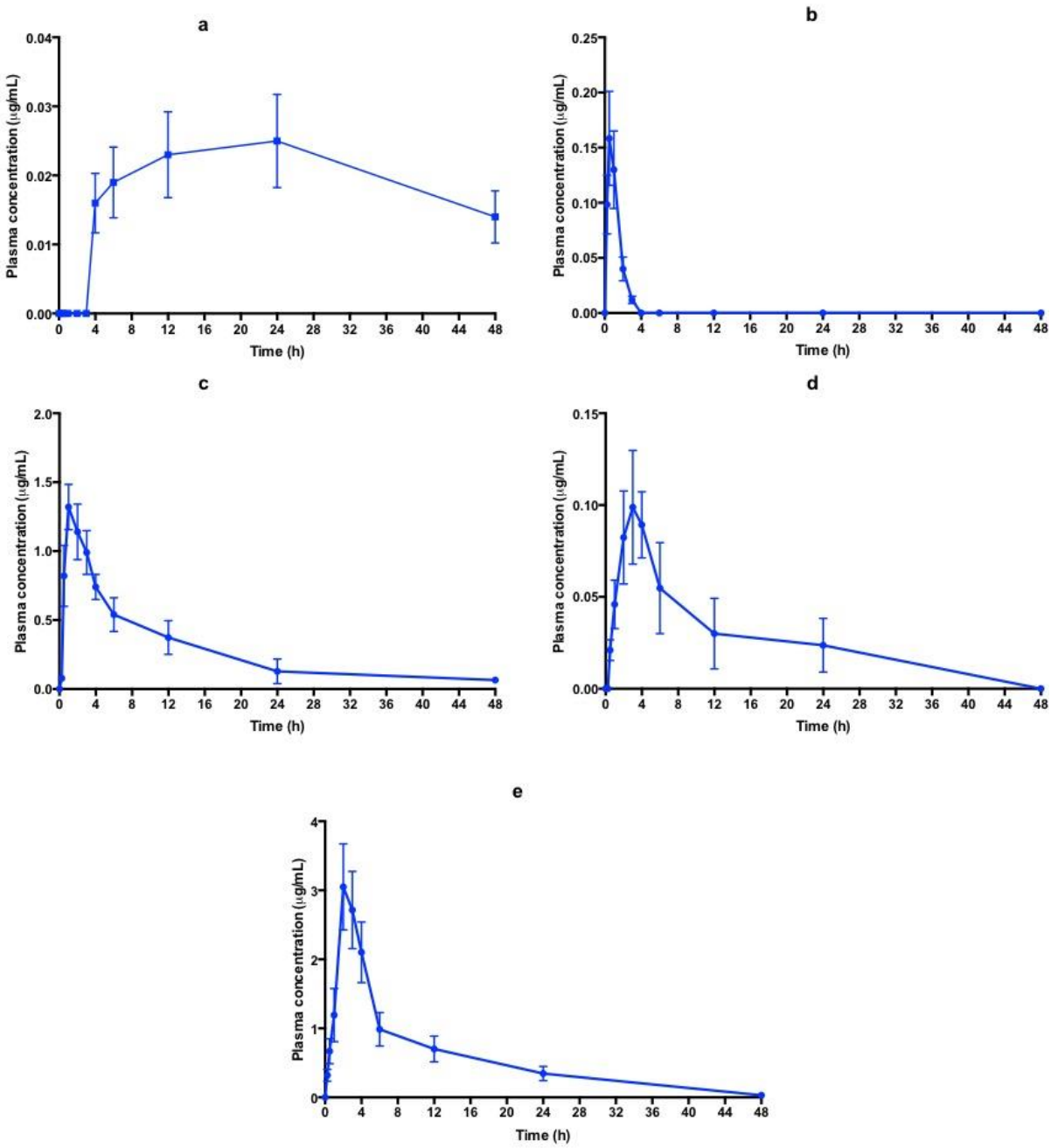

Figure 3. The mean plasma concentration and time profile of IVM (a), ABZ (b), ABZ-OX (c), ABZ-ON (d) and DOX (e) after oral administration (means ± SD, n = 6 for each group).

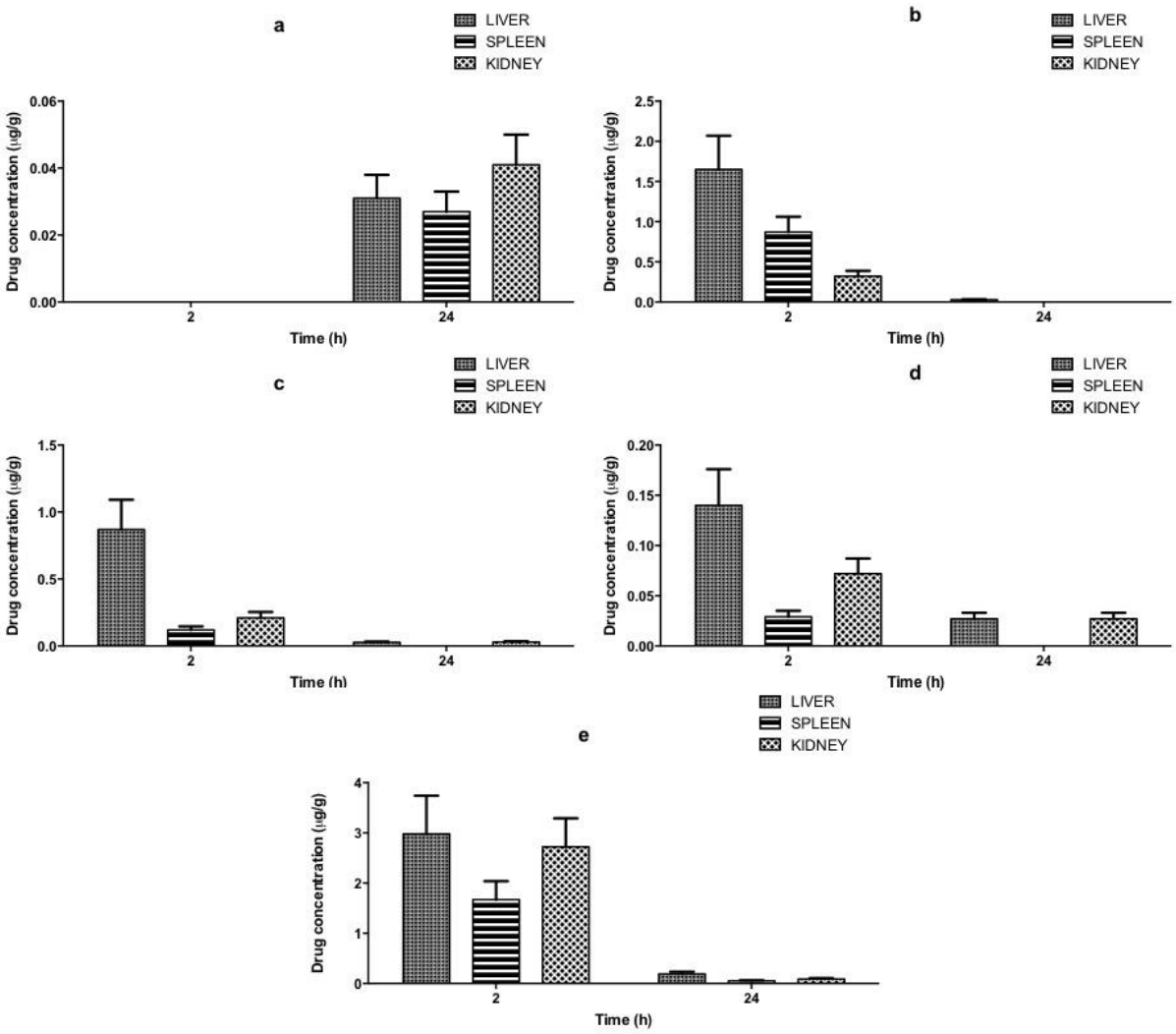

Figure 4. Biodistribution profile of IVM (a), ABZ (b), ABZ-OX (c), ABZ-ON (d) and DOX (e) in the liver, the spleen and the kidney after oral administration (n = 6 for each group).

Table 1. Gradient conditions for HPLC-UV mobile phase.

Time	A (%)	B (%)	UV detection (nm)
0-5	75	25	270
5-12	75	25	290
12-14	30	70	245
14-18	5	95	245
18-20	75	25	270

Table 2. Properties of the calibration curve for quantification of all analyes with LOD and LLOQ values.

	Analyte	Slope	y-intercept	R	LOD (µg/mL)	LLOQ (µg/mL)
Plasma	IVM	1036.8	-8.43	0.999	0.006	0.01
	ABZ	409.03	-6.77	0.999	0.01	0.03
	ABZ-OX	432.54	-8.75	0.998	0.009	0.03
	ABZ-ON	414.52	-7.32	0.998	0.008	0.03
	DOX	403.76	-8.16	0.999	0.01	0.03
Liver	IVM	1102.4	-8.26	0.999	0.007	0.01
	ABZ	414.98	-6.63	0.998	0.009	0.03
	ABZ-OX	403.19	-8.93	0.998	0.009	0.03
	ABZ-ON	423.42	-7.47	0.998	0.009	0.03
	DOX	418.32	-8.32	0.998	0.01	0.03
Spleen	IVM	1021.2	-7.35	0.999	0.007	0.01
	ABZ	419.71	-5.90	0.998	0.009	0.03
	ABZ-OX	409.12	-7.94	0.999	0.01	0.03
	ABZ-ON	432.12	-7.62	0.999	0.008	0.03
	DOX	411.10	-8.49	0.998	0.009	0.03
Kidney	IVM	1114.2	-7.50	0.999	0.006	0.01
	ABZ	400.34	-6.03	0.999	0.009	0.03
	ABZ-OX	419.12	-7.79	0.998	0.009	0.03
	ABZ-ON	409.13	-7.47	0.998	0.01	0.03
	DOX	403.42	-8.32	0.999	0.01	0.03

Table 3. Intra- and Inter-day precision and accuracy of IVM, ABZ, ABZ-OX, ABZ-ON and DOX in rat plasma (n = 6).

		Intra-day			Inter-day		
Analyte	**Concentration added (µg/mL)**	**Concentration found (µg/mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**	**Concentration found (µg/mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
IVM	0.01	0.01 ± 0.00	2.32	3.21	0.01 ± 0.00	3.25	3.65
	0.03	0.03 ± 0.00	3.12	6.67	0.03 ± 0.00	7.14	-6.67
	15	15.32 ± 0.53	3.46	2.13	14.12 ± 0.41	2.90	-5.87
	37.5	39.91 ± 1.21	3.03	6.43	35.95 ± 2.02	5.61	-4.13
ABZ	0.03	0.03 ± 0.00	4.17	6.97	0.03 ± 0.00	8.13	-7.62
	0.08	0.07 ± 0.00	2.74	-2.67	0.07 ± 0.01	8.45	-5.33
	25	23.01 ± 1.03	4.48	-7.96	27.53 ± 1.54	5.59	10.12
	75	76.08 ± 2.87	3.77	1.44	77.01 ± 3.81	4.95	2.68
ABZ-OX	0.03	0.03 ± 0.00	5.12	4.91	0.03 ± 0.00	3.15	-2.61
	0.08	0.08 ± 0.00	2.47	8.00	0.08 ± 0.01	6.10	9.33
	25	26.45 ± 1.87	7.07	5.80	27.09 ± 2.12	7.83	8.36
	75	74.21 ± 3.02	4.07	-1.05	77.25 ± 2.19	2.83	3.00
ABZ-ON	0.03	0.03 ± 0.00	5.42	4.91	0.03 ± 0.00	3.18	-4.12
	0.08	0.07 ± 0.00	1.35	-1.33	0.08 ± 0.01	4.94	8.00
	25	26.43 ± 2.01	7.60	5.72	27.41 ± 2.91	10.62	9.64
	75	76.92 ± 3.81	4.95	2.56	77.01 ± 3.33	4.32	2.68
DOX	0.03	0.03 ± 0.00	9.41	4.31	0.03 ± 0.00	2.58	-3.92
	0.08	0.08 ± 0.00	2.5	6.67	0.08 ± 0.01	5.06	5.33
	25	27.12 ± 1.15	4.24	8.48	23.11 ± 2.15	9.30	-7.56
	75	74.98 ± 3.21	4.28	-0.03	77.18 ± 4.21	5.45	2.91

Table 4. Intra- and Inter-day precision and accuracy of IVM, ABZ, ABZ-OX, ABZ-ON and DOX in rat liver (n = 6).

		Intra-day			Inter-day		
Analyte	**Concentration added (µg/mL)**	**Concentration found (µg/mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**	**Concentration found (µg/mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
IVM	0.01	0.01 ± 0.00	4.12	2.81	0.01 ± 0.00	2.78	4.13
	0.03	0.03 ± 0.00	4.09	-3.33	0.03 ± 0.00	5.03	-10.7
	15	13.98 ± 0.63	4.53	-6.80	12.98 ± 0.72	5.58	-13.5
	37.5	33.16 ± 1.32	3.97	-11.57	34.09 ± 1.66	4.88	-9.1
ABZ	0.03	0.03 ± 0.00	4.11	3.92	0.03 ± 0.00	3.73	8.12
	0.08	0.07 ± 0.01	3.59	-2.67	0.07 ± 0.00	4.41	-10.1
	25	23.91 ± 1.40	5.87	-4.36	25.75 ± 1.86	7.22	3
	75	73.12 ± 3.61	4.94	-2.51	81.90 ± 4.98	6.07	9.2
ABZ-OX	0.03	0.03 ± 0.00	6.15	3.21	0.03 ± 0.00	4.32	5.32
	0.08	0.08 ± 0.01	3.24	9.33	0.07 ± 0.01	3.98	-10.7
	25	27.09 ± 2.51	9.26	8.36	22.95 ± 2.61	11.39	-8.2
	75	72.98 ± 3.89	5.33	-2.69	84.75 ± 5.56	6.56	13
ABZ-ON	0.03	0.03 ± 0.00	3.21	3.41	0.03 ± 0.00	4.11	-3.41
	0.08	0.07 ± 0.00	1.77	-8.00	0.07 ± 0.00	2.18	-9.8
	25	27.67 ± 2.76	9.96	10.68	27.45 ± 3.36	12.25	9.8
	75	72.72 ± 4.72	6.48	-3.04	67.43 ± 5.38	7.98	-10.1
DOX	0.03	0.03 ± 0.00	8.11	3.18	0.03 ± 0.00	3.12	6.17
	0.08	0.07 ± 0.00	3.28	-2.67	0.07 ± 0.00	4.03	-1.8
	25	26.19 ± 1.46	5.55	4.76	22.80 ± 1.56	6.83	-8.8
	75	73.21 ± 4.11	5.61	-2.39	81.90 ± 5.65	6.90	9.2

41 **Table 5.** Intra- and Inter-day precision and accuracy of IVM, ABZ, ABZ-OX, ABZ-ON and DOX in rat spleen (n = 6).

		Intra-day			Inter-day		
Analyte	**Concentration added (µg/mL)**	**Concentration found (µg/mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**	**Concentration found (µg/mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
IVM	0.01	0.01 ± 0.00	8.10	2.82	0.01 ± 0.00	1.89	3.83
	0.03	0.03 ± 0.00	6.74	1.50	0.03 ± 0.00	5.85	-14.63
	15	14.68 ± 1.09	7.48	-2.14	13.21 ± 1.66	12.53	-11.93
	37.5	34.82 ± 2.28	6.55	-7.15	32.43 ± 1.84	5.68	-13.52
ABZ	0.03	0.03 ± 0.00	4.17	-5.15	0.03 ± 0.00	4.17	-5.19
	0.08	0.08 ± 0.01	5.92	2.20	0.06 ± 0.00	5.14	-14.67
	25	25.11 ± 2.43	9.68	0.42	24.05 ± 2.02	8.40	-3.80
	75	76.78 ± 6.26	8.15	2.37	76.49 ± 5.41	7.07	1.99
ABZ-OX	0.03	0.03 ± 0.00	7.16	4.02	0.03 ± 0.00	-8.23	9.13
	0.08	0.09 ± 0.01	5.34	14.80	0.07 ± 0.00	4.63	-8.00
	25	28.44 ± 4.07	14.32	13.78	21.44 ±2.84	13.26	-14.26
	75	76.63 ± 6.74	8.80	2.17	79.16 ± 6.04	7.63	5.54
ABZ-ON	0.03	0.03 ± 0.00	4.19	-4.16	0.03 ± 0.00	4.19	-7.73
	0.08	0.07 ± 0.00	2.92	-3.40	0.07 ± 0.01	2.53	-13.33
	25	28.05 ± 3.18	11.33	12.20	25.64 ± 2.16	8.43	2.55
	75	76.36 ± 10.70	10.70	1.81	68.64 ± 6.37	9.28	-8.48
DOX	0.03	0.03 ± 0.00	8.18	-4.75	0.03 ± 0.00	5.19	-8.19
	0.08	0.08 ± 0.01	5.40	2.20	0.07 ± 0.00	4.69	-8.28
	25	27.50 ± 2.52	9.16	10.00	21.30 ± 1.69	7.95	-14.82
	75	76.87 ± 7.11	9.25	2.49	76.49 ± 6.14	8.03	1.99

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Table 6. Intra- and Inter-day precision and accuracy of IVM, ABZ, ABZ-OX, ABZ-ON and DOX in rat kidney (n = 6).

		Intra-day			Inter-day		
Analyte	**Concentration added (µg/mL)**	**Concentration found (µg/mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**	**Concentration found (µg/mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
IVM	0.01	0.01 ± 0.00	3.42	-1.81	0.01 ± 0.00	4.69	-3.13
	0.03	0.03 ± 0.00	6.83	3.29	0.03 ± 0.00	7.40	13.44
	15	15.98 ± 1.04	6.51	6.56	16.32 ± 0.87	5.32	8.80
	37.5	39.24 ± 1.56	3.98	4.64	35.42 ± 2.54	7.18	-5.55
ABZ	0.03	0.03 ± 0.00	3.19	8.28	0.03 ± 0.00	7.13	8.21
	0.08	0.08 ± 0.00	5.15	3.25	0.09 ± 0.01	6.50	13.39
	25	27.43 ± 2.31	8.42	9.72	23.32 ± 2.15	9.21	-6.72
	75	80.98 ± 5.74	7.09	7.97	78.43± 7.01	8.94	4.57
ABZ-OX	0.03	0.03 ± 0.00	6.19	-3.11	0.03 ± 0.00	3.19	-4.01
	0.08	0.08 ± 0.01	4.64	11.32	0.08 ± 0.01	5.86	8.00
	25	25.94 ± 3.23	12.46	3.75	28.48 ± 4.77	16.76	13.93
	75	73.21 ± 5.60	7.65	-2.39	80.40 ± 7.76	9.65	7.20
ABZ-ON	0.03	0.03 ± 0.00	9.13	-4.11	0.03 ± 0.00	5.11	6.11
	0.08	0.08 ± 0.01	2.54	4.87	0.08 ± 0.01	3.20	10.67
	25	27.87 ± 3.98	14.29	11.48	21.36 ± 2.28	10.66	-14.56
	75	83.05 ± 7.73	9.31	10.74	78.98 ± 9.27	11.73	5.31
DOX	0.03	0.03 ± 0.00	8.15	6.15	0.03 ± 0.00	5.43	-7.72
	0.08	0.08 ± 0.01	4.70	10.98	0.07 ± 0.01	5.93	-9.33
	25	25.77 ± 2.06	7.97	3.07	28.30 ± 3.74	13.21	13.19
	75	81.43 ± 6.55	8.05	8.57	79.09 ± 8.03	10.15	5.45

Table 7. Mean extraction recoveries of IVM, ABZ, ABZ-OX, ABZ-ON and DOX in rat plasma (n=6).

Analyte	Concentration added (µg/mL)	% Extraction Recovery ± SD	% RSD
IVM	0.01	67.93 ± 5.98	8.80
	0.03	69.01 ± 6.09	8.82
	15	65.98 ± 8.05	12.20
	37.5	74.91 ± 8.87	11.84
ABZ	0.03	93.21 ± 8.32	8.93
	0.08	87.76 ± 7.19	8.19
	25	95.19 ± 6.53	6.86
	75	93.87 ± 8.13	8.67
ABZ-OX	0.03	85.33 ± 7.54	8.84
	0.08	87.98 ± 8.43	9.58
	25	83.42 ± 8.34	9.99
	75	91.17 ± 9.52	10.44
ABZ-ON	0.03	87.98 ± 5.84	6.64
	0.08	89.92 ± 9.98	11.09
	25	83.19 ± 7.21	9.07
	75	79.45 ± 8.45	10.63
DOX	0.03	97.21 ± 9.98	10.27
	0.08	96.12 ± 7.41	7.71
	25	100.32 ± 10.43	10.39
	75	98.23 ± 8.98	9.14

Table 8. Mean extraction recoveries of IVM, ABZ, ABZ-OX, ABZ-ON and DOX in rat liver (n=6).

Analyte	Concentration added (µg/mL)	% Extraction Recovery ± SD	% RSD
IVM	0.01	70.98 ± 5.86	8.26
	0.03	71.03 ± 6.36	8.95
	15	67.93 ± 4.37	6.44
	37.5	73.23 ± 6.36	8.69
ABZ	0.03	90.54 ± 8.54	9.43
	0.08	83.21 ± 6.54	7.86
	25	93.24 ± 10.39	11.14
	75	91.98 ± 9.95	10.81
ABZ-OX	0.03	90.21 ± 8.92	9.89
	0.08	88.77 ± 6.29	7.09
	25	83.41 ± 11.02	13.21
	75	92.54 ± 10.80	11.67
ABZ-ON	0.03	89.54 ± 8.14	9.09
	0.08	91.32 ± 3.54	3.87
	25	79.43 ± 10.24	12.89
	75	81.09 ± 11.51	14.20
DOX	0.03	99.21 ± 8.32	8.39
	0.08	98.32 ± 7.05	7.17
	25	99.32 ± 13.00	13.09
	75	101.09 ± 12.41	12.28

Table 9. Mean extraction recoveries of IVM, ABZ, ABZ-OX, ABZ-ON and DOX in rat spleen (n=6).

Analyte	Concentration added (µg/mL)	% Extraction Recovery ± SD	% RSD
IVM	0.01	80.43 ± 7.64	9.49
	0.03	73.30 ± 8.86	12.09
	15	74.72 ±5.83	7.80
	37.5	82.02 ± 3.91	4.77
ABZ	0.03	93.21 ± 5.43	5.83
	0.08	90.70 ± 5.60	6.17
	25	96.22 ± 9.71	10.09
	75	99.61 ± 8.46	8.49
ABZ-OX	0.03	93.18 ± 8.65	9.28
	0.08	91.61 ± 5.10	5.57
	25	85.16 ± 12.71	14.93
	75	96.52 ± 8.85	9.17
ABZ-ON	0.03	93.19 ± 9.54	10.23
	0.08	94.61 ± 2.88	3.04
	25	81.97 ± 6.54	7.98
	75	83.60 ± 9.32	11.15
DOX	0.03	100.21 ± 9.54	9.52
	0.08	100.68 ± 5.67	5.63
	25	101.60 ± 9.71	9.55
	75	99.07 ± 9.55	9.64

Table 10. Mean extraction recoveries of IVM, ABZ, ABZ-OX, ABZ-ON and DOX in rat kidney (n=6).

Analyte	Concentration added (µg/mL)	% Extraction Recovery ± SD	% RSD
IVM	0.01	85.17 ± 8.14	9.56
	0.03	76.45 ± 8.18	10.70
	15	76.29 ± 9.05	11.87
	37.5	89.15 ± 9.27	10.39
ABZ	0.03	97.01 ± 8.32	8.58
	0.08	96.23 ± 9.04	9.40
	25	97.47 ± 12.18	12.49
	75	97.62 ± 12.62	12.93
ABZ-OX	0.03	94.76 ± 8.32	8.78
	0.08	89.78 ± 7.61	8.47
	25	88.82 ± 7.37	8.30
	75	98.55 ± 13.76	13.96
ABZ-ON	0.03	93.43 ± 9.02	9.65
	0.08	98.68 ± 4.57	4.63
	25	82.96 ± 8.42	8.43
	75	86.28 ± 10.53	12.21
DOX	0.03	93.18 ± 8.34	8.95
	0.08	98.23 ± 8.42	8.58
	25	99.01 ± 14.40	14.54
	75	97.09 ± 14.25	14.68

Table 11. Mean stability recoveries of IVM, ABZ, ABZ-OX, ABZ-ON and DOX at different storage conditions (n=3).

		% Stability recoveries (mean ± SD)			
	Analyte	**Autosampler (48 h)**	**Bench-top (24 h)**	**Long-term (2 weeks)**	**Freeze-thaw (3 cycles)**
Plasma	IVM	101.03 ± 8.54	98.18 ± 4.65	100.93 ± 9.87	98.92 ± 6.43
	ABZ	99.43 ± 7.98	97.79 ± 8.27	101.33 ± 8.62	96.27 ± 8.33
	ABZ-OX	97.98 ± 6.87	98.91 ± 7.98	98.21 ± 8.76	98.21 ± 8.43
	ABZ-ON	99.87 ± 9.32	98.31 ± 9.98	99.28 ± 8.82	97.32 ± 9.01
	DOX	98.43 ± 9.11	97.81 ± 2.55	98.89 ± 7.65	97.98 ± 2.49
Liver	IVM	99.98 ± 3.43	98.87 ± 7.32	98.38 ± 7.43	98.91 ± 9.03
	ABZ	97.81 ± 8.32	95.89 ± 9.27	99.29 ± 7.32	98.13 ± 4.32
	ABZ-OX	97.18 ± 8.87	97.98 ± 8.98	97.91 ± 7.09	99.28 ± 2.09
	ABZ-ON	98.81 ± 4.52	99.98 ± 4.32	99.21 ± 4.59	98.81 ± 5.64
	DOX	100.98 ± 8.12	100.32 ± 5.43	100.31 ± 9.73	99.08 ± 8.45
Spleen	IVM	98.76 ± 8.19	98.92 ± 9.87	99.29 ± 9.04	96.53 ± 4.94
	ABZ	97.28 ± 8.23	97.21 ± 5.43	97.23 ± 3.43	97.43 ± 9.03
	ABZ-OX	99.02 ± 6.98	98.91 ± 8.98	96.98 ± 8.43	98.81 ± 9.93
	ABZ-ON	100.19 ± 10.01	96.45 ± 6.76	97.39 ± 2.98	98.98 ± 5.43
	DOX	98.81 ± 3.12	99.23 ± 3.91	98.39 ± 8.98	97.87 ± 3.21
Kidney	IVM	97.16 ± 7.62	98.87 ± 7.54	98.93 ± 2.65	98.13 ± 3.98
	ABZ	96.87 ± 8.09	99.89 ± 9.32	99.09 ± 8.73	97.09 ± 4.08
	ABZ-OX	99.17 ± 2.39	97.65 ± 3.54	100.32 ± 10.21	98.07 ± 3.89
	ABZ-ON	100.87 ± 10.01	98.13 ± 3.98	98.82 ± 4.39	96.92 ± 3.54
	DOX	98.64 ± 8.83	98.91 ± 7.65	99.91 ± 8.65	97.98 ± 6.49

Table 12. Pharmacokinetics parameters of IVM, ABZ, ABZ-OX, ABZ-ON and DOX after oral administration in Wistar rats (means ± SD, n = 6 for each group).

Parameters	IVM	ABZ	ABZ-OX	ABZ-ON	DOX
Dose (mg/kg)	0.4	15	-	-	10
C_{max} (µg/mL)	0.026 ± 0.01	0.16 ± 0.03	1.32 ± 0.32	0.10 ± 0.02	3.05 ± 0.79
T_{max} (h)	24	0.5	1	3	2
AUC_{0-t} (µg/mLh)	1.08 ± 0.23	0.24 ± 0.05	13.16 ± 3.12	0.99 ± 0.23	26.99 ± 5.67
AUC_{0-INF} (µg/mLh)	1.08 ± 0.19	0.24 ± 0.05	14.35 ± 2.76	1.34 ± 0.28	27.35 ± 5.98
$T_{1/2}$ (h)	10.72 ± 2.58	2.99 ± 0.71	12.69 ± 2.61	10.38 ± 2.98	8.24 ± 1.98

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