Determination Of Ivermectin, Albendazole & Diethyl Carbamazine Citrate By Using LCMS/MS

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Abstract

An accurate LC-MS/MS method was established and validated to study the pharmacokinetic properties of Ivermectin (IVER) Albendazole (ALB) & Diethylcarbamazine (DEC) in the plasma of rat using carbamazepine (CBZ) as internal standard (IS). For the extraction of a combination of IVER, ALB, and DEC from rat plasma, the protein precipitation method was used. The monitored MS/MS ion transitions were 897.6 → 753.4 for IVER, 200 →127 for DEC, 266.1→234 for ALB, and, 237→ 194 for Carbamazepine. The method's lower limit of quantitation (LLOQ) was 11.41 ng/ml for ALB and DEC and for IVER, it was 46.87ng/ ml in rat plasma with a mean recovery of 81.22%. for ALB, 80.24%. for DEC and 77.66%. for IVER. The fine chromatographic separation and resolution of peaks have been attained with 25 mM Ammonium formate in water and 0.1 % formic acid in methanol on the Kinetex Evo C18 column. The total run time for the method was 7 minutes. The method was found to be accurate and precise with a linearity range of 46.87 ng/mL to 24000 ng/ mL for IVER and 11.71 ng/mL to 6000 ng/ mL for ALB as well as DEC with a correlation coefficient (r) of 0.9977, 0.9990, 0.9994 respectively.

Keywords: MS/MS analysis; Ivermectin; Albendazole; Diethyl carbamazine; rat plasma, LCMS/MS method

INTRODUCTION

Lymphatic filariasis, a disease caused by filarial parasites it is also called as elephantiasis. This disease is dangerous as the adult form of the parasite can survive up to 6 to 8 years in the lymphatic system of humans. There it produces many microfilariae, immature filaria which circulates throughout the blood. The adult also hampers the host's lymphatic system, impairing the immune system. When any mosquito bites an infected patient, this microfilaria enters the mosquito's body

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where it grows into mature filaria which further transfers to the healthy host with a mosquito bite. After entering in healthy host's lymphatic system and starts growing into an adult. Hence this vicious circle continues. In 2000, the WHO launched the Global Program to Eliminate Lymphatic Filariasis (GPELF) to interrupt LF transmission. Each subject at risk of contracting the disease is given either a single drug (diethylcarbamazine, DEC) or a combination of drugs (DEC, ALB, IVR) also known as MDA (mass drug administration). Efforts to be made by the WHO to administer drugs. Recent studies [(Kalyanasundaram R. et.al. (2016), Ottesen, E. A. (2006), Thomsen E. K. (2016), Peter U et al. (2017), Ediet al (2019), P. Jambulingam P. et al. (2021), King C.L. et al (2018), M. Hardy et al (2020), Tripathi B. et al.(2022)] indicate that a triple-drug remedy, a mixture of IVER, ALB & DEC, is more effectively leading to encouragement for MDA (Mass drug management) regime in nations without onchocerciasis i.e., IVER 200 µg, ALB 400 mg, and DEC 6 mg per kg in certain settings are administered.

Drug profile

An invertebrate's cell membrane is flooded with chloride ions by IVER. This results in hyperpolarization leading to muscle paralysis. (Richard J et al, 2021)

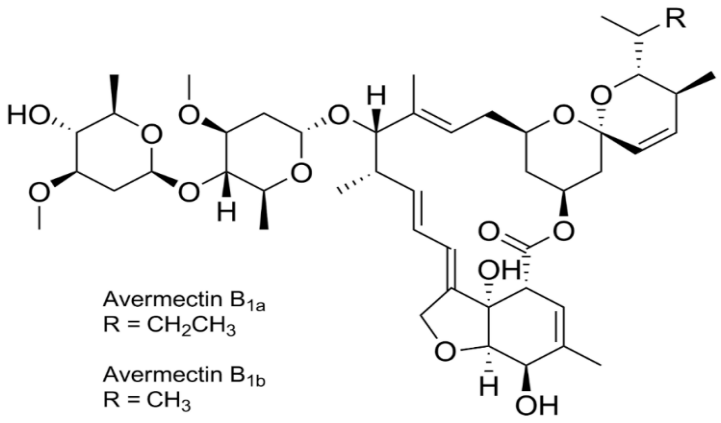


Fig. 1: IVER

IUPAC name: IVER is a mixture containing at least 90% 5-Odemethyl-22,23-dihydroavermectin A1a and less than 10% 5-O-demethyl-25-de(1-methylpropyl)-22,23-dihydro25-(1-methylethyl) avermectin A1a , generally referred to as 22,23-dihydroavermectin B1a and B1b, or H2B1a and H2B1b, respectively.

Tegmental and intestinal cells of intestinal helminths and larvae suffer from specific degeneration of microtubules by ALB. Inhibition of

microtubule polymerization occurs due to the metabolite's binding to B-tubulin subunits of microtubules of the helminth. (Kashif Malik et al)

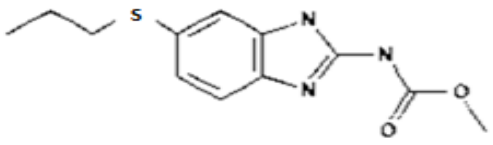


Fig. 2: ALB

IUPAC NAME: methyl N-(6-propylsulfanyl-1H-benzimidazol-2-yl) carbamate

The mechanism of diethylcarbamazine is related to sensitizing the microfilaria to phagocytosis. According to research, its effectiveness against Burgia malai microfilaria relies on the presence of inducible nitric oxide synthases and the cyclooxygenase pathway. DEC targets cyclooxygenase pathway, COX-1, and 5-lympho-oxygenase pathway through the arachidonic acid metabolic pathway, thus playing an important role in DEC mechanism of action. (McGarry, H. F 2023)

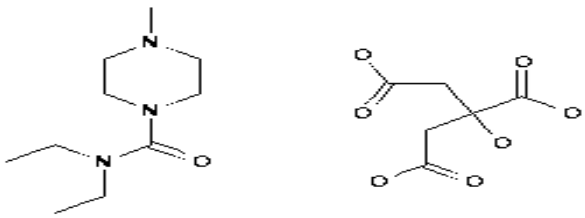


Fig. 3: DEC

IUPAC name: N,N-diethyl-4-methylpiperazine-1-carboxamide;2-hydroxypropane-1,2,3-tricarboxylic acid

Not many validated methods are available for quantifying all three drugs together in the biological matrix. Thus, a bioanalytical method has been developed and validated in this work for the quantification of IVER ALB & DEC for further studies.

Chemicals and reagents

IVER and ALB standards were obtained from Vivan life science whereas DEC was acquired from Shubham Pharmaceuticals. Internal standard (Carbamazepine) was purchased from Vivan life science. For the mobile phase preparations LCMS Grade methanol, and pure water were procured from J.T. Baker Chemicals. Formic acid and ammonium formate was procured from RANKEM and Sigma Aldrich respectively. Blank rat plasma was collected at Sphaera Pharma Pvt Ltd, Animal laboratory (IMT Manesar).

Instrumentation and chromatographic conditions

The analysis was performed on AB Sciex triple quad 6500+ (ESI) coupled with Nexra LC-40 UPLC. The UPLC column Kinetex EVO C18 (100 mm × 4.6 mm, 2.6 µm) was used for good chromatographic separations. The mobile phase used for pumps A and B was 25 mM ammonium formate in water and 0.1% formic acid in methanol respectively. Gradient elution was performed with a flow rate of 0.600 ml/min and 2µL of samples were injected. 90% Methanol and 10% of water were used as needle wash. The column compartment and autosampler temperature were maintained at 30^{o}C and 10^{o}C respectively.

Table 1: Gradient Program

Time	Concentration of A (%)	Concentration of B (%)
0.00	30	70
2.50	05	95
4.00	05	95
6.00	30	70
7.00	30	70

All the data acquisition and quantification were managed by Analyst® (version 1.7.2) software. To increase the sensitivity of the multiple reactions monitoring (MRM), a concentration of 100 ng/ml of all analytes and internal standard was infused for calibrating the molecule. The spectrometer was configured to operate in MRM mode, with both quadruples set to unit resolution.

TABLE 2: MS parameters

	Q1 Mass (Da)	Q3 Mass (Da)	DP(volts)	EP(volts)	CE(volts)	CXP(volts)
Dec	200.1	127.1	36	10	21	14
Iver	897.6	753.4	200	10	60	10
Alb	266.1	234.0	146	10	27	14
Cbz	236.0	194.1	11	10	27	10

Sample preparation

The extraction of IVR, ALB, and DEC from the plasma protein precipitation method was used. 25µL of plasma was added in a 1.5ml Eppendorf, and 5µL of carbamazepine (25ng/ml) was added as internal standard and mixed on vibramax for 30 sec. Then 500µL of reconstitution solution (80% of methanol with 0.1% of Formic acid: 20% of 25mM ammonium formate in water) was added and Eppendorf was vibramaxed for 5 min, after that kept for centrifugation at 5,000 rpm for 10 min at 4˚C (Eppendorf 5804 R). The upper organic layer was collected with the help of a pipette and transferred to labeled HPLC vials for further analysis.

Method Validation

The validation of the method followed the guidelines set by ICH, ensuring its credibility and accuracy. Q2 (R1) pertains to linearity, accuracy, precision, matrix effect, autosampler carryover test, stability and recovery.

Calibration Curve and Linearity: Fresh aqueous standards for CCs were prepared by repeating multiple serial dilutions. A regression model involves limited or no weighing (using 1/x^2). The tests were performed according to the established standards to check the linearity. The ratio of peak area of the analyte to the peak area of the internal standard was plotted to calculate the calibration curve.

Carryover and Selectivity: Carryover test was performed to ensure there is no carryover to next sample, in order to quantify the exact concentration of samples. Selectivity test is a way to verify interference in peak. Six typical plasma samples were collected which is having one lipidemic and one haemolysed sample. Blanks and LLOQs were performed with those plasma samples. The accepted limit for interference at analyte peak is less than 20% of area of the analyte of LLOQ and less than 5% of the internal standard area.

Precision and accuracy: Precision states the proximity of closeness of agreement among a sequence of experiments obtained from multiple sampling of identical samples under the same circumstances. Accuracy, also known as trueness, signifies the proximity of consensus among the actual value and the discovered value. The precision was determined using percentage CV (coefficient of variation). Four different concentrations i.e., LLOQ, LQC (lower quality control), MQC (middle-quality control), and HQC (higher quality control) were used to determine the method's precision and accuracy. To assess the

accuracy, the calculated concentration value obtained from the calibration curve was compared to the nominal concentration value. In order to meet the accuracy requirements, the relative standard deviation from the nominal value should be under ±15%, ensuring that the accuracy falls within the range of 85–115%.

Recovery: Recovery of analytes was calculated at three different concentration levels (LQC, MQC, and HQC) by comparing the area of peak of each analyte in extracted QCs (Quality Controls) samples with post-extracted QCs. To calculate the recovery of analyte and internal standard overall mean recovery, standard deviation, and % CV were required to calculate.

Stability: Assessment of stability must be conducted to guarantee that each measure was taken throughout sample preparation and sample analysis, as well as the storage conditions utilized, do not impact the concentration of the analyte. Stabilities were analysed at various different conditions for validation such as autosampler stability, bench-top stability, freeze-thaw stability, and wet extract stability (RT and RF). Stabilities were also carried out in replicates (n=6) with LQC and HQC concentrations. To analyse the stability nominal concentrations of samples were compared with freshly prepared samples of the same concentrations.

RESULTS

Mass spectrum

Mass spectrum of IVER $[M+H]^+$ m/z was 897.6, for ALB $[M + H]^+$ m/z was 266.1, for Diethyl carbamazine $[M + H]^+$ m/z was 200.1 and for carbamazepine $[M + H]^+$ m/z was 237. A full positive mode scan was done to enhance ESI settings for IVER, ALB, DEC, and CBZ. Product ion employed for IVER, ALB, and DEC were 753.4, 234.0 and 127.1 respectively. Similarly, for carbamazepine, the product ion of 194.1 was employed.

Selectivity and carryover

The blank plasma sample showed zero peak area at the RT of all analytes as well as internal standard. Similarly, blanks injected after ULOQ (upper limit of quantification) was also not showing any peak area. Hence, the result suggests there is no carryover. Selectivity was assessed by evaluating all six lots of plasma on the instrument. No substantial interferences were observed at the RT of analytes as well as RT of internal standard.

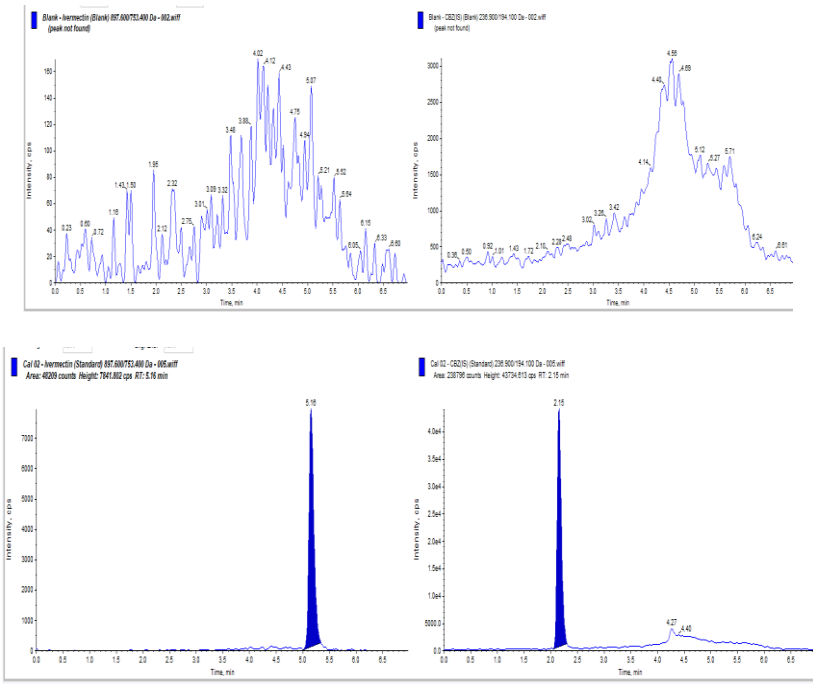


Fig.4.MRM spectrum: (A) blank rat plasma, (B) blank rat plasma with Ivermectin and carbamazepine

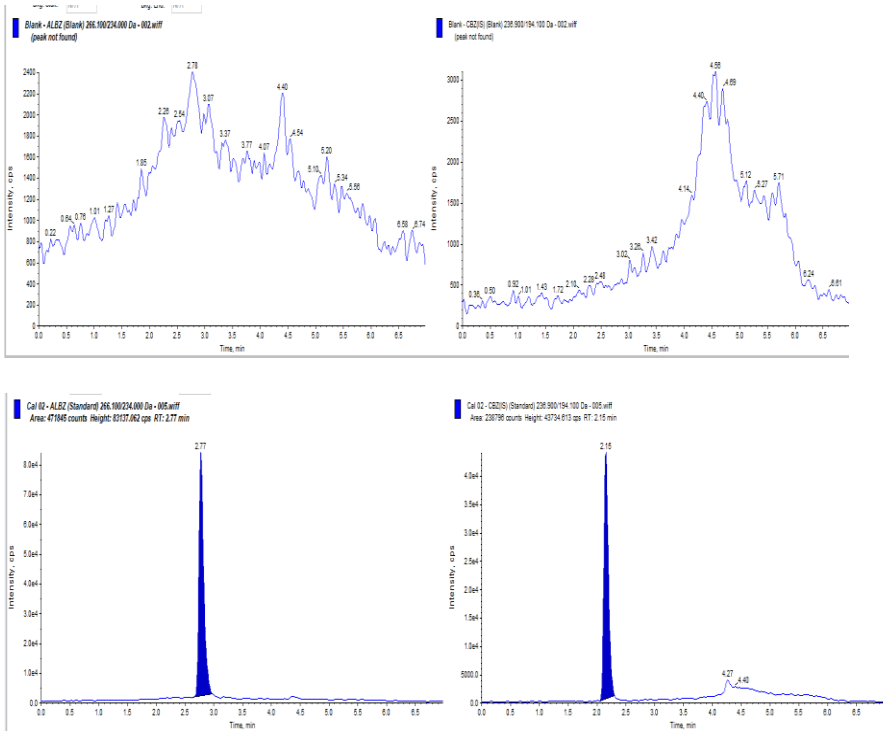


Fig.5.MRM spectrum: (A) blank rat plasma, (B) blank rat plasma with Albendazole and carbamazepine

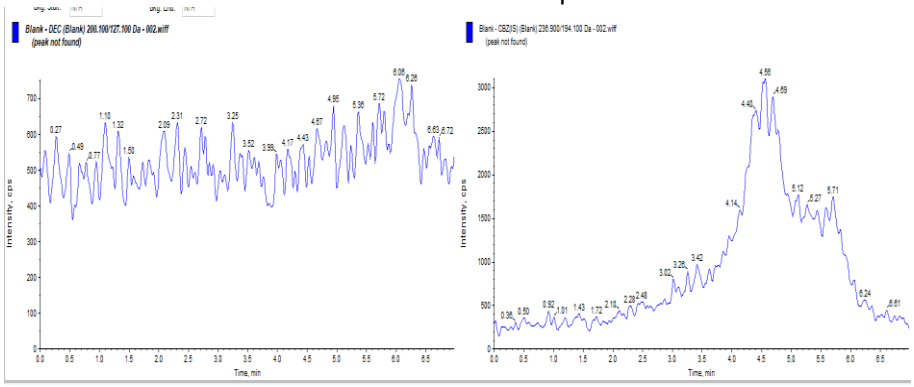
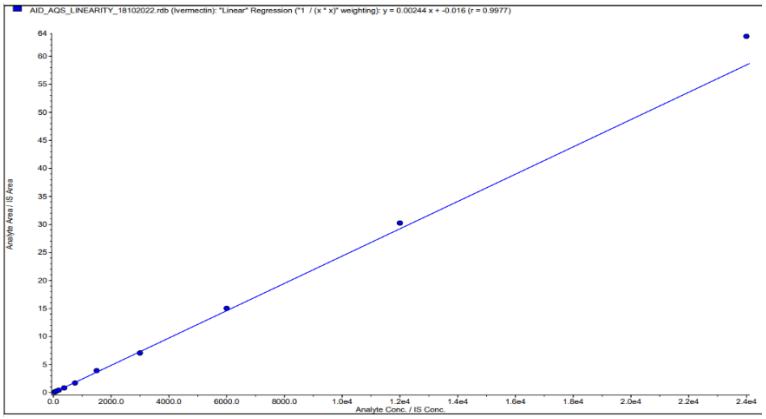


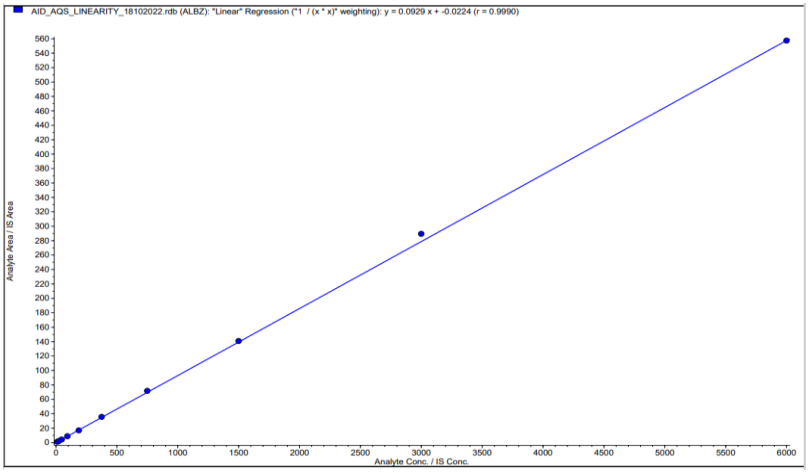
Fig.6.MRM spectrum: (A) blank rat plasma, (B) blank rat plasma with DEC and carbamazepine

3.3 Calibration curve and linearity

The linearity was constructed by making a ten-point standard calibration curve in rat plasma. The linearity range for IVER was 46.87 ng/mL to 24000 ng/ mL, for ALB as well as DEC it was 11.71 ng/mL to 6000ng/ mL. The correlation coefficient r = 0.9977 for IVER, 0.9990 for ALB and 0.9994 for DEC. The slope values were assessed by a weighting factor of $1/x^2$ calibration curve standards.



(A)



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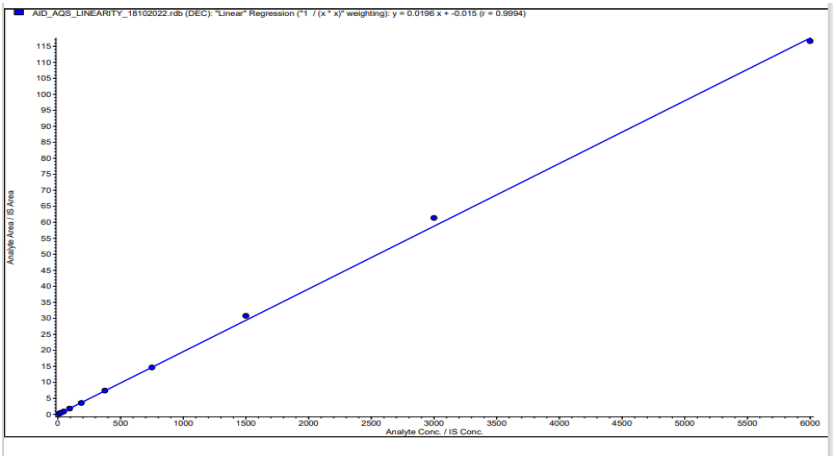

(C)

Fig.7. Mean of the ten standard curves in plasma used in method validation by LC-MS/MS using carbamazepine as internal standard

(25 ng/mL) for (A) Ivermectin, (B) Albendazole and (C) Diethylcarbamazine.

Precision and accuracy

The accuracy and precision of all three analytes for intra-day and inter-day at the quality control samples were within the acceptable limits with %CV values for ALB was 1.0–2.8% (intra-day) and 1.1–5.8% (inter-day), for IVER it was 2.8-8.1% (intra-day) and 3.2–8.1% (inter-day) and for DEC vales were 1.5–5.9% (intra-day) and 1.9–5.8% (inter-day). The intra- and inter-day accuracy precision results are summarized in table 3.

Table 3. Precision and accuracy of LCMS/MS analysis in rat plasma

		ALB				DEC				IVER			
		HQC	MQC	LQC	LLOQC	HQC	MQC	LQC	LLOQC	HQC	MQC	LQC	LLOQC
		3000	750	46.87	11.71	3000	750	46.9	11.71	12000	3000	187.5	46.87
		Nominal Concentration (ng/mL)				Nominal Concentration (ng/mL)				Nominal Concentration (ng/mL)			
PNA_1	SD	48.1	9.9	0.5	0.2	65.3	12.7	1.2	0.7	711.6	92.7	5.4	2.0
	% CV	1.7	1.2	1.0	2.0	2.2	1.5	2.5	5.9	6.0	2.8	2.8	3.8
	% ACCURACY	94.1	108.9	101.6	102.7	101.2	111.7	98.1	94.4	99.2	109.2	102.2	113.6
PNA_2	SD	48.2	21.8	0.8	0.3	89.2	28.8	0.9	0.3	431.9	266	11.3	2.0
	% CV	1.6	2.8	1.8	2.6	2.6	3.9	2.1	2.2	3.2	8.1	5.3	3.8
	% ACCURACY	100.0	103.0	93.0	99.6	113.6	98.5	94.3	103.3	113.7	108.9	114.9	116.3
PNA_3	SD	81.8	8.6	0.7	0.7	141.3	14.3	1.0	0.6	517.6	235.4	6.3	2.8
	% CV	2.7	1.1	1.5	5.8	4.1	1.9	2.3	5.8	4.2	7.5	3.4	5.3
	% ACCURACY	100.2	106.3	99.9	100.0	115.0	102.4	96.2	90.2	101.8	104.1	99.3	111.8

Recovery

The peak area ratios in plasma samples are compared to the peak area ratios added to blank plasma extract to measure extraction recovery. Table 4. shows the recovery of IVER as 82.91%, 77.75%, and 72.32% for HQC, MQC, and LQC respectively. Similarly, in the case of ALB, it is 83.39 % 81.03%, and 79.24 % and DEC is 87.35%, 82.54%, and 70.45 %. The total mean recovery for IVER is 77.66%., ALB is 81.22% and DEC is 80.11%.

Table 4. Recovery in rat plasma (QCs)

	ALBENDAZOLE				DEC			IVERMECTIN		
	ANALYTE RECOVERY									
		EXTRACTED	AQS	RECOVERY	EXTRACTED	AQS	RECOVERY	EXTRACTED	AQS	RECOVERY
HQC	AVG	70558928.5	84615640.5	**83.4**	13942249.0	15961036.7	**87.4**	9094633.8	10968674.7	82.9
	SD	1976674.0	440797.3		460631.9	203268.7		413805.3	294685.7	
	%CV	2.8	0.5		3.3	1.3		4.6	2.7	

MQC	AVG	17378746.0	21448006.0	**81.0**	2875138.7	3483452.2	**82.5**	2148356.2	2763161.5	77.8
	SD	297014.8	229113.5		64623.0	20497.9		157752.7	98691.6	
	%CV	1.7	1.1		2.3	0.6		7.3	3.6	
LQC	AVG	987801.7	1246594.0	**79.2**	162279.2	230360.8	**70.5**	114816.3	158750.7	72.3
	SD	7750.5	18215.3		4240.6	5312.9		4525.8	6303.4	
	%CV	0.8	1.5		2.6	2.3		3.9	4.0	

Matrix Effect

In the course of simultaneous method validation of IVER, ALB, and DEC in plasma, the matrix effect was calculated by evaluating LQC and HQC. All analytes as well as internal standard did not show any matrix effect. The average matrix effect is based on the ratio of the response of post-spiked concentrations to the aqueous standard response. Results are given in Table 5.

Table 5. Matrix effect in rat plasma

	ALB		DEC		IVER		CBZ		IS NORMALISED MATRIX FACTOR	
S.No.	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC
1	1.04	1.03	1.01	1.00	0.92	0.82	1.01	1.05	1.03	0.98
2	1.02	0.99	1.00	0.97	0.96	0.85	0.98	1.00	1.04	0.99
3	1.01	1.01	1.00	0.95	0.94	0.77	0.96	1.01	1.05	1.00
4	1.02	0.99	1.02	0.97	0.94	0.87	1.02	0.97	1.00	1.02
5	1.00	0.98	0.99	0.99	0.93	0.83	0.98	1.01	1.02	0.97
6	1.01	0.97	1.01	1.00	0.97	0.97	1.00	1.00	1.01	0.97
AVERAGE									1.03	0.99
SD									0.02	0.02
% CV									1.82	1.96

Stability

The stability was assessed at two concentrations i.e., LQC and HQC. It was estimated on the basis of few parameters such as autosampler stability (36 hr), bench-top stability (6 hr), freeze/thaw at –80°C (three cycles), and wet extract stability at RF and RT (49 hr). See the results in Table 6. All results were found to be within the assay variability range.

Table 6. Stability of sample at different conditions in rat plasma

Sample conditions	ALBENDAZOLE (ALB)				DEC				IVERMECTIN			
	Nominal concentration (ng/ml)				Nominal concentration (ng/ml)				Nominal concentration (ng/ml)			
	Observed %	% CV	Observed %	% CV	Observed %	% CV	Observed %	% CV	Observed %	% CV	Observed %	% CV
	46.87 ng/ml (LQC)		3000 ng/ml (HQC)		46.87 ng/ml (LQC)		3000 ng/ml (HQC)		187.50 ng/ml (LQC)		12000 ng/ml (HQC)	
Freshly prepared	102.87	1.59	111.2	1.65	99.11	2.35	108.36	0.68	93.66	3.81	104.03	3.03
Bench-top stability (6 hr)	106.23	1.86	113.9	1.72	98.26	2.37	107.91	1.41	107.1	3.84	108.29	2.37
Autosampler stability (36 hr)	108.59	2.97	113.68	0.49	99.82	3.51	108.09	2.05	105.45	2.41	114.26	2.1
Wet extract stability at RF (49 hr)	101.76	2.05	107.01	1.89	98.88	2.51	110.69	2.37	110.8	2.02	114.93	2.32
Wet extract stability at RT (49 hr)	100.76	2.6	107.95	1.87	96.82	2.66	109.19	2.29	99.46	3.45	112.87	3.4
Three freeze-thaw cycles	105.49	2.21	111.31	1.14	98.45	0.91	106.3	1.48	107.58	3.42	114.7	3.32

Conclusion

A combination of all three drugs, IVER, Albendazole, and diethyl carbamazine citrate, is under MDA trial for a treatment for Lymphatic filariasis. This method of quantification LCMS/MS analysis is highly sensitive, concise, and precise. Extraction of analytes from plasma was done with the protein precipitation method. The method is time-saving and cost-effective. The validated method was successfully applied to pharmacokinetic studies.

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