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RP- HPLC method for the determination of Ivermectin and Clorsulon as Active Pharmaceutical Ingredients in goat plasma

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Abstract: Background: Ivermectin and Clorsulon are anthelmintic compounds that are active against types of parasites that are used as an injectable mixture or as a single injectable product for cattle, sheep, horses, and goats. To identify and determine ivermectin and clorsulon as active pharmaceutical ingredients in spiking goat plasma, a linear RP-HPLC method was designed and validated. This approach is precise, simple, accurate, selective, and linear. Separation was performed with a mobile phase composed of a mixture of acetonitrile: ethanol: water (52: 28: 20 v/v %), respectively, at flow rate 1.2 mL/min on column Luna C8 column (4.6 mm, 250 mm, 5µm, 100oA) thermostated at 25o C with UV detection at 254 nm. The diluent is a mixture of mobile phase: methanol (75:25 v/v %). As APIs, the method has been validated for linearity, accuracy, precision, and robustness. The calibration graph was linear in the range of 950-3.8 µg/mL, 50-0.5 µg/mL and 500-1.25 µg/mL for IVMH2B1a, IVM H2B1b and CLR, respectively with regression coefficient (R2) equal 0.999 for each one, with percentage accuracy was 99.42 ± 0.43% for IVM H2B1a, 101.2± 0.05% for IVMH2B1b and 99.46 ± 0.66% for CLR. Among the analytical methods for estimation ivermectin and clorsulon in plasma require a laborious clean-up step on an SPE cartridge; the work is based on simple and rapid liquid-liquid extraction utilizing water-soluble organic solvents. Using a blank plasma sample spiked with IVM and CLR and underwent the method. It was linear in range of 475-2.4 µg/mL, 25-0.5 µg/mL and 250-2.5 µg/mL for IVMH2B1a, IVM H2B1b and CLR, respectively with percentage recovery was 97.98 ± 0.41 %for IVM H2B1a, 97.7 ± 0.15% for IVMH2B1b and 100.27 ± 0.23% for CLR and the intra- and inter-day precision with RSD% did not exceed 2%. Therefore, the proposed method can be used for routine analysis of ivermectin and clorsulon.

Keywords: *HPLC, determination, ivermectin, clorsulon.*

Introduction

Ivermectin (IVM) (Fig.1a) is an anthelmintic compound active against numerous types of parasites in domestic animals [1, 2]. The effectiveness of IVM as an antiparasitic agent for sheep, pigs, and cattle is attributed to its potency [3, 4]. Figure 1 shows that IVM is a chemical combination of 22,23-dihydro avermectin B1a and 22,23-dihydro avermectin B1b, two ivermectin derivatives [1, 3, 5, 6]. Ivermectin is a class of highly active broad-spectrum anti-parasitic agents isolated from the fermentation products of Streptomyces avermitilis [7].

Onchocerciasis and strongyloidiasis are two human parasitic diseases treated with IVM, an antiparasitic drug that has FDA approval for tablet form [8]. External parasites like head lice and skin disorders like rosacea can be effectively treated with topical versions of IVM. Reportedly, human-use items differ from animal-use ones and are only safe for use in animals when directed to do so by a veterinarian [9].

The chemical formula for chlorsulfuron (CLR) is 4-amino-6-(1,2,2-trichloroethenyl)-benzene-1,3-disulfonamide (Fig. 1b). Oral or subcutaneous use of the medicine is effective against adult flukes. Cattle infected with adult liver (Fasciola hepatica, Fasciola gigantica) should be treated and managed with CLR in an injectable or suspension form [5, 10]. Typically, it is mixed with IVM and applied to livestock such as sheep, goats, and cattle [10]. The active pharmaceutical ingredients (APIs) in the injectable product number around 1% IVM and 10% CLR, respectively, in terms of weight percent by volume. Combining IVM and CLR into one injectable solution offers the best of both worlds for parasite control and prevention in cattle [11].

Several chromatographic techniques have been documented for the estimation of these active pharmaceutical ingredients (APIs) both alone and in mixtures with other medications. Consequently, establishing a unified chromatographic approach for the detection and quantification of IVM and CLR is not a simple task [11, 12]. The announced approach utilizes distinct HPLC procedures for each component in the United States Pharmacopeia (USP) monograph [13] as a single compound or as an injectable form. For IVM as a single API, the USP reported a method where the mobile phase was a mixture of acetonitrile, methanol, and water (53:27.5:19.5), using methanol as a diluent with (4.5 mmx25 cm contains 5 µm packing) C18 column [13] and for CLR as a single API mobile phase is a mixture of acetonitrile, methanol, and water (53:27.5:19.5 v/v) using methanol as diluent with (4.5 mmx25 cm contains 5 µm packing) C18 column [13] and for CLR as a single API, the mobile phase is a mixture of water, acetonitrile, and glacial acetic acid (70:30:0.1 v/v) using the mobile phase as diluent with (4.5 mmx25 cm contains 5 µm packing) C8 column [14]. These two methods may be tiresome for QC laboratories where these methods are not rapid for qualitative and quantitative analysis of IVM and CLR.

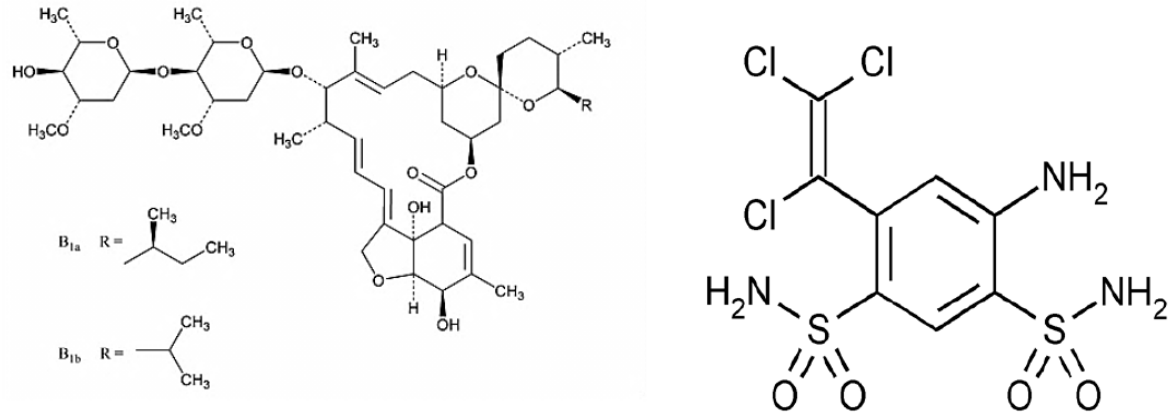


Figure 1: Structure formula of ivermectin (a) and clorsulon (b).

The main purpose of designing the method is the identification and determination of IVM and CLR in bio-fluid samples, entire blood, plasma, serum, urine, and other bodily fluids are all considered biological fluids. Proteins are abundant in the three main components of blood, which are whole blood, plasma, and serum. [15, 16]. It is critical to remove these proteins before analysis. Accordingly, different methods of sample preparation have been applied to improve protein removal from plasma to ease the extraction of target compounds from a bio-fluid sample which involves liquid-liquid extraction (LLE), organic solvent precipitation, as well as solid-phase

extraction (SPE) [17, 18]. The most common problems with solid-phase extraction (SPE) are that it is expensive and sample extracts are insufficiently clean, so solvent precipitation and liquid-liquid extraction are recommended because it is well-suited for high-throughput sample analysis, take little time at all, and don't cost a fortune to prepare samples [17]. It is expected that better protein removal leads to improved extraction since the applied matrix is goat plasma.

Comparing the effectiveness of protein removal of four classes including an organic solvent, acid, salt, and metal in plasma by Polson et al, found that, the most efficient organic solvents were acetonitrile: ethanol: methanol with ability of 93.2%: 88.6%: 88.7% protein removal, respectively [17, 19]. With a 2:1 solvent-to-serum ratio, Want and colleagues compared 14 protein precipitation tests (PPT) in serum, including organic precipitation, acid precipitation, and heat denaturation. The results showed that methanol had a 98% protein removal capacity, while ethanol had a 96% ability [17, 20].

The hydration layer of proteins is destroyed, the repulsive forces between protein molecules are reduced, and the solubility of proteins is decreased when water-miscible solvents such as acetonitrile, methanol, ethanol, or a combination of these are used. This results in the precipitation of proteins, which can be removed by filtration and centrifugation, and the supernatant can be used for analysis [21, 22]. Utilization of acetonitrile for PPT gives large coagulated yellow precipitates, while methanol and ethanol give finer coagulated white precipitates. The clarity and hazy supernatant refers to protein precipitation efficiency, the more clear the more good protein precipitation [17, 23, 24].

This study established and verified a novel approach in accordance with the ICH Q2 (R1) standard. [25]. A straightforward RP-HPLC technique that is both sensitive and selective for identifying and estimating IVM and CLR in bulk and spiked plasma matrices; the approach is also accurate, precise, and robust. For the examination of IVM and CLR as bulk APIs, this recently designed and verified RP-HPLC method would be ideal for use in quality control QC laboratories. For the first time, we present a new mobile phase and diluent. For both IVM and CLR analyses, it demonstrated a shorter $t_{R.}$ Introducing a new diluent used for PPT in the matrix sample and extracting the two drugs in one step, applying the clear and clean supernatant in the HPLC system without requiring (SPE) purification.

2. Experimental

2.1. Apparatus

In chromatographic analysis, a Thermoscientific UltiMate 3000 HPLC system was utilized. The system included a DIONEX UltiMate 3000 pump, an autosampler, a column compartment, and a variable wavelength UVS detector. For data processing, Chromeleon version 7.2 was used from Thermoscientific. An ultrasonic bath from Elma Schmidbaver GmbH, a four-digit balance from Sartorius, and a pH meter from METTLER TOLEDO were used.

2.2 Materials and reagents

We only employed analytical-grade chemicals and reagents, and HPLC-grade solvents, in our experiments. The CLR (99%) and IVM (99%) working standards were generously provided by El-Nasr Pharmaceutical Chemicals Co., El-Salam City, Egypt. To separate the samples, a nylon membrane filter (0.45 µm) from Phenomenex (USA) was utilized. Sigma-Aldrich of Germany supplied the labeled HPLC-grade ethanol, methanol, and acetonitrile with a purity level of 98.5%. The entire process relied on bi-distilled water.

2.3. Chromatographic conditions

Luna C8 column (4.6 mm, 250 mm, 5µm,100^{o}A) from Phenomenex thermostated at 25^{o}C and mobile phase consisted of acetonitrile: ethanol: water (52:28:20) v/v were used. The mobile phase was filtrated through a 0.45µm millipore membrane filter and degassed for 15 minutes by sonication before use. The diluent consisted of a mobile phase: methanol (75:25), respectively. The flow rate of the system was 1.2 mL/min with an injected volume of 20 µL and the wavelength of the detector at 254 nm.

2.4. Standard solutions

A 1 mg/mL IVM stock solution and a 0.5 mg/mL CLR stock solution were made in diluent. To create working solutions in the concentration ranges of 1000 -4µg/mL for IVM and 500-1.25µg/mL for CLR, the stock solutions were diluted with the same diluent. We refrigerated all of the solutions until we needed them.

2.5. Method validation

Linearity, limit of detection (LOD), limit of quantification (LOQ), selectivity, precision, accuracy, and robustness were all evaluated in accordance with International Conference Harmonization (ICH) standards [25].

2.5.1. System suitability

Using five replicate injections of the working standard, system suitability characteristics were obtained. The relative standard deviation of peak regions, tailing factor, plate count, and resolution were the parameters.

2.5.2. Linearity

The linearity of the method was studied by plotting the response (area under the peak) versus the injected concentration over the concentration range of 950-3.8 µg/mL for IVM H_2B_{1a}, the concentration range of 50-0.5 µg/mL of IVM H_2B_{1b} and concentration range of 500-1.25 µg/mL of CLR.

2.5.3. Limit of detection (LOD) and limit of quantification (LOQ)

Detection and quantification limits described the method's sensitivity as (LOD) = (3.3 σ/S) and (LOQ) = (10σ/S), where σ is the response's standard deviation (SD) and S is the slope of the calibration curve.

2.5.4. Selectivity

The chromatograms showed no overlapping peaks, which allowed us to study selectivity without influence from the shared suspension or mobile phase.

2.5.5. Precision and Accuracy

Repeatability and intermediate precision were used to verify the method's precision and accuracy. Measurements were taken triplicately (n=3) at three distinct concentrations, covering the concentration range of the calibration curve, to assess repeatability.

2.5.6. Robustness

How well a method handles small changes to its typical operating settings is one way to measure its robustness. Some examples of such factors are variations in column temperature, mobile phase pH, mobile phase composition (even small changes), and flow rate.

2.6. Sample preparation and extraction

To 5 mL blank plasma sample, at room temperature, 50 mg of IVM which containing 47.5 mg of IVM H_2b_{1a} and 2.5 mg of IVM H_2b_{1b}, and 25 mg of CLR were added and mixed well by shaking and sonicated for 10 minutes. The mixture was transferred to a 100 mL volumetric flask containing 50 ml of the diluent, allowed to stand for 5 minutes then shaken and sonicated for 10 minutes. The volume was completed to 100 mL with the same diluent and sonicated for 15 minutes at 40oC. It was allow standing until the temperature of the solution reached temperature and the precipitate by filtration through a 0.45µm millipore membrane.

3. Result and discussion

3.1. Analytical method development:

To measure IVM and CLR concurrently, the HPLC method was devised as a stability-indicating technique. At a temperature of 25˚C, a Luna C8 column measuring 4.6 mm, 250 mm, 5µm, and 100oA was employed. The development phase included experimenting with various flow rates and acetonitrile to ethanol-to-water ratios. We selected a mobile phase consisting of acetonitrile, ethanol, and water in the proportion of 52:28:20 (v/v) and a flow rate of 1.2 mL/min based on the results and chromatographic characteristics. In different ratios, methanol: ethanol, methanol: water, ethanol: water, and mobile phase were tried as diluents. The best diluent was mobile phase: methanol in a ratio of 75:25 v/v. At this condition, IVM and CLR were eluted.The selected wavelength was 254nm, which showed a good response for both IVM and CLR, Figure 2.

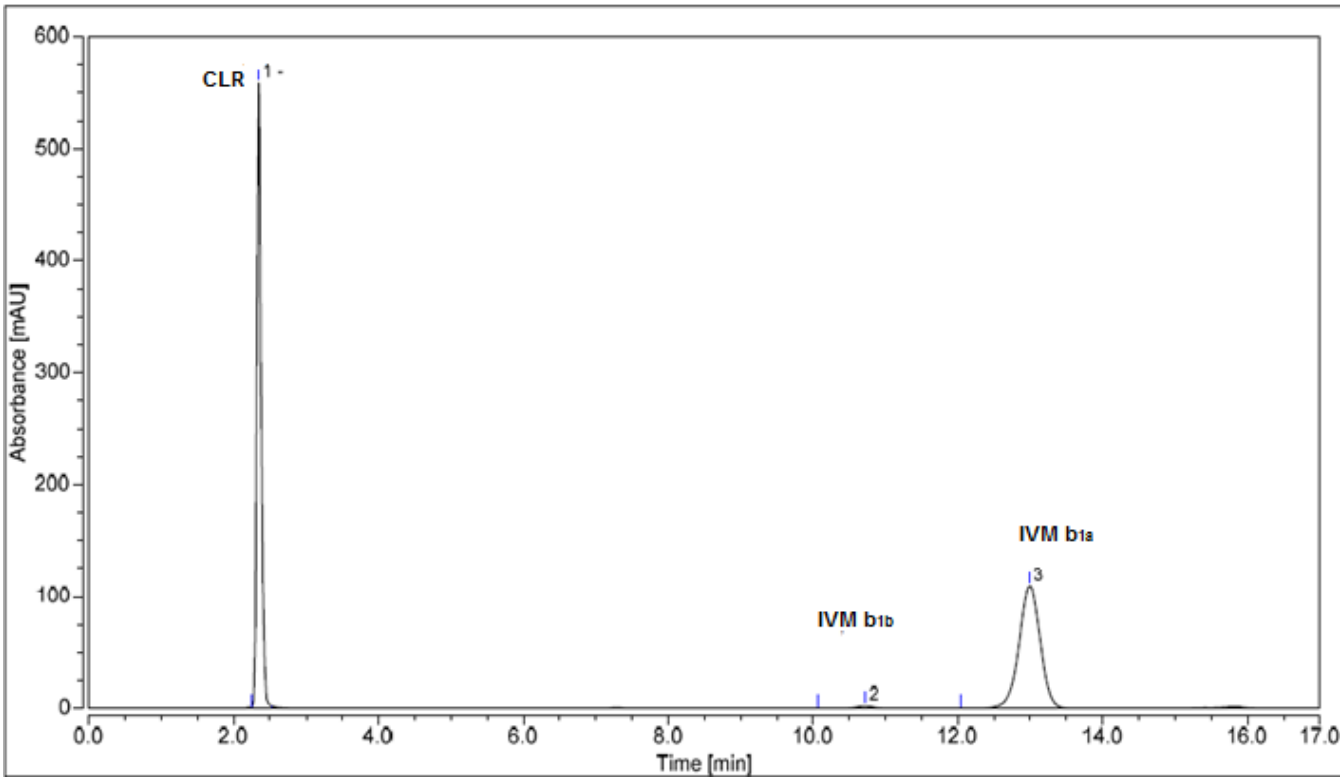


Figure 2: The IVM and CLR chromatogram, produced under ideal chromatographic circumstances.

3.2. System suitability

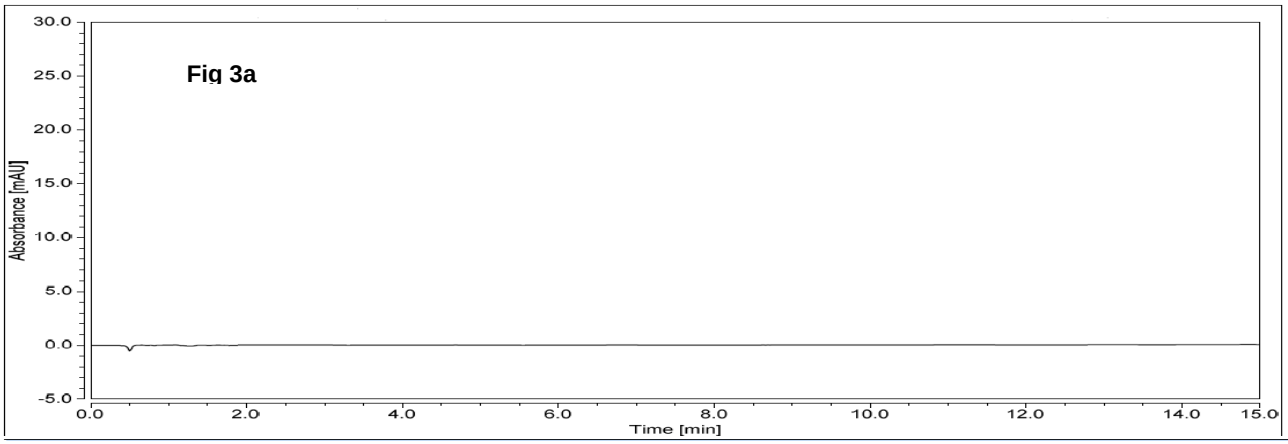
Table 1 shows the results of the system suitability tests for IVM and CLR at a concentration of 0.05 mg/mL. The system is doing well since the values are within the acceptable range.

Table 1: Chromatographic characteristics of system suitability

Parameter	IVM		CLR	Recommended limit
	H_2B_{1a}	H_2B_{1b}		
Peak Area	**17.26**	**0.53**	**19.55**	**-**
Retention time	**12.9**	**10.71**	**2.34**	**-**
Resolution	**33.79**	**31.35**	**-**	**> 1.5**
Plate Count	**9604**	**9473**	**8140**	**> 2000**
Tailing factor	**0.96**	**0.81**	**1.29**	**≤ 2**

3.3. Selectivity

The mobile phase blank and diluent HPLC chromatograms are typical examples, as shown in Figures 3a and b, respectively. Figure 4a shows the injection of the IVM sample solution and Figure 4b shows the injection of the CLR sample solution. The mobile phase blank and diluent blank HPLC chromatograms that were recorded did not show any peaks at the retention times of IVM and CLR. Good selectivity of the technique was demonstrated by the consistency of retention durations of IVM and CLR between the mobile phase solution and the diluent solution.



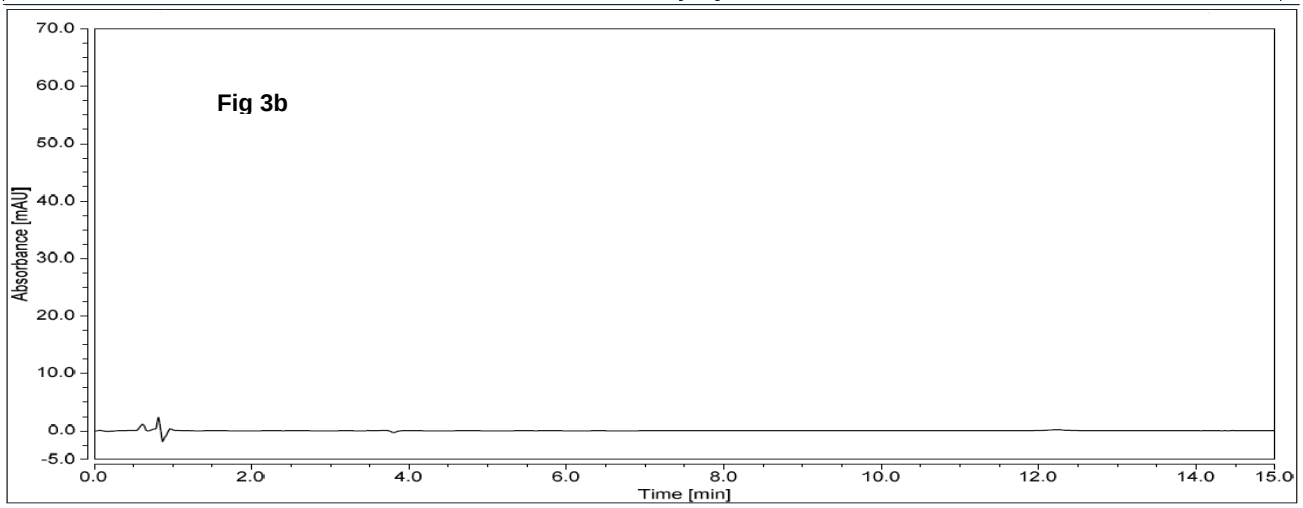
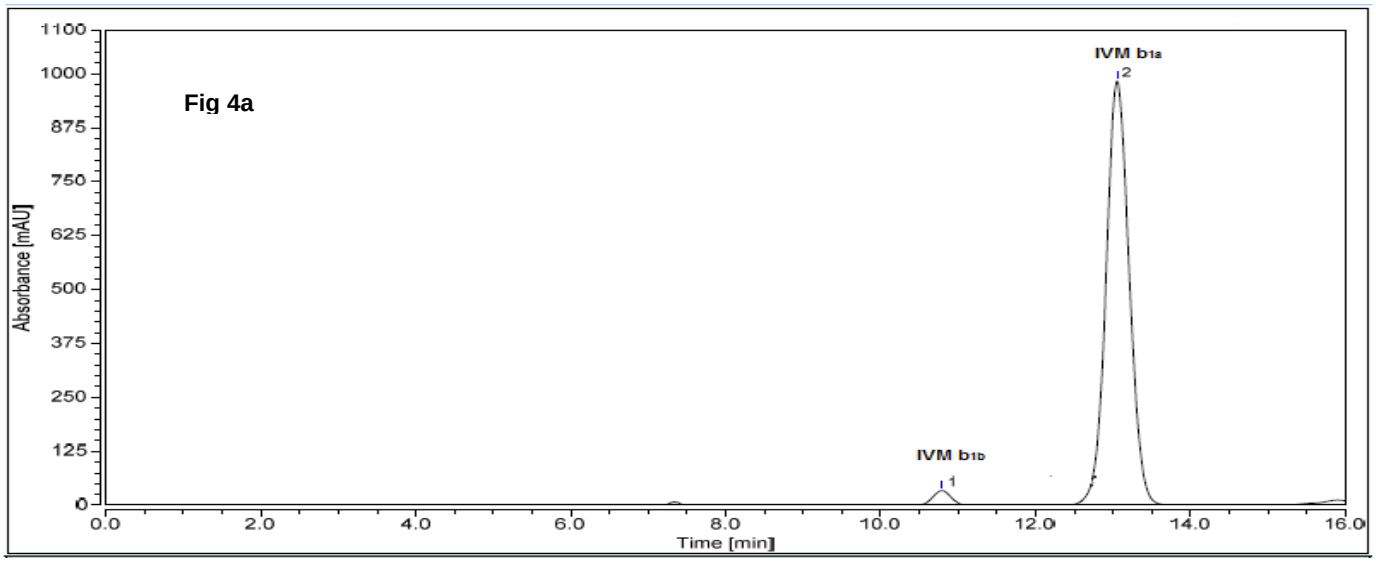


Figure 3: Chromatogram of mobile phase blank (a), Chromatogram of diluent blank (b).



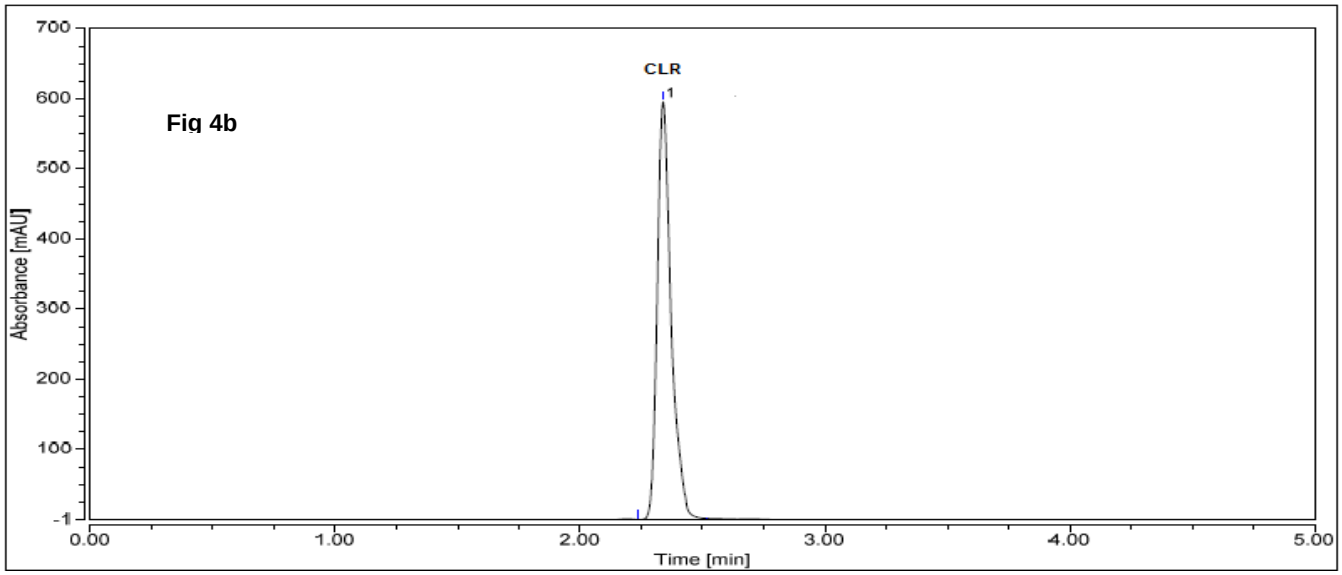
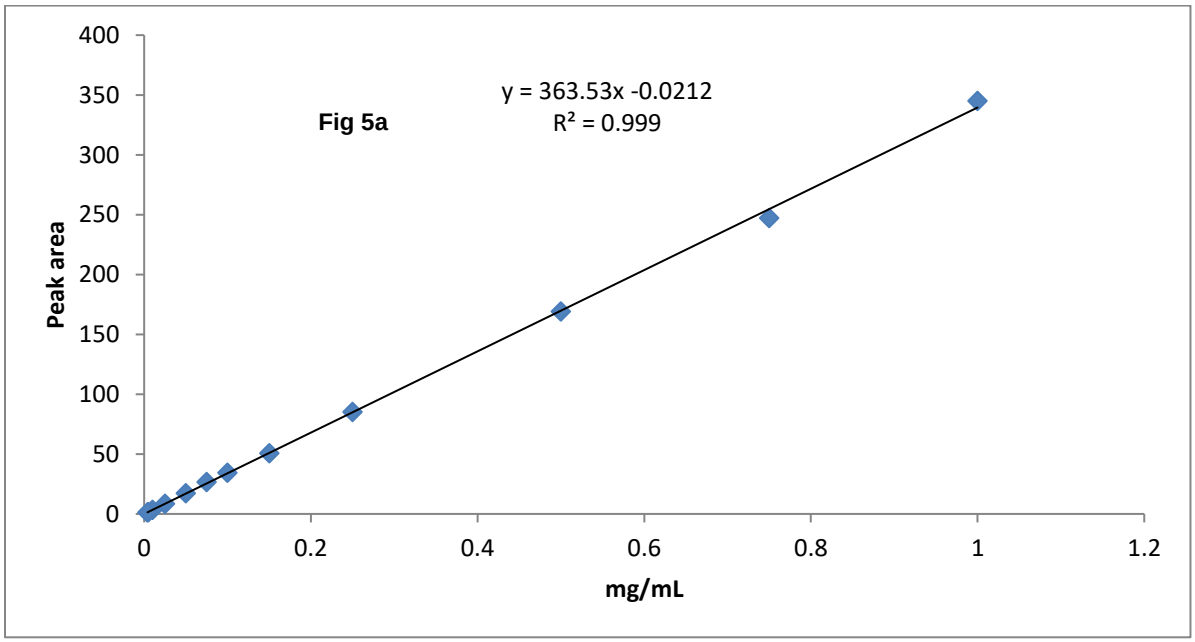
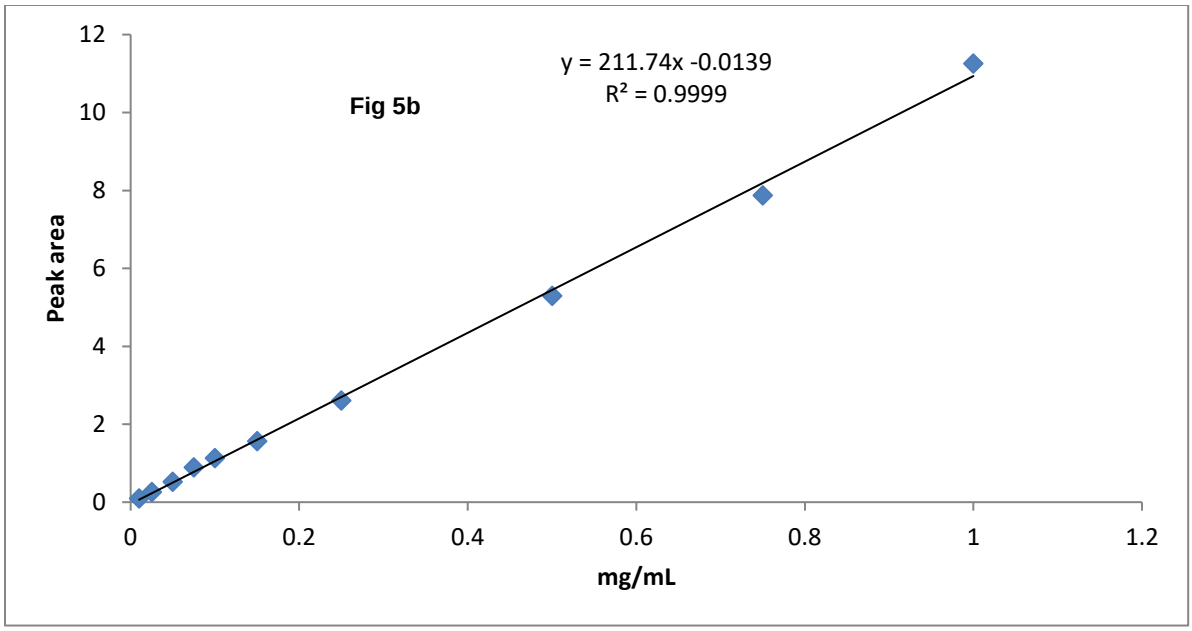


Figure 4: Chromatogram of (a) CLR and (b) IVM using acetonitrile: ethanol: water in the ratio of 52:28:20 (v/v) as a mobile phase with a flow rate of 1.2 mL/min at 254 nm.

3.4. Linearity

Using the regression equation (y = 363.53x -0.0212) and the regression coefficient (R2) 0.999, the linearity for IVMH2B1a was found to be between 950 and 3.8 µg/mL when graphing peak area (y) against concentration (x). A linearity of 50-0.5 µg/mL was observed for IVMH2B1b, with a regression equation of y = 211.74x - 0.0139 and a regression coefficient (R^2) of 0.9999. The linearity coefficient (R2) for CLR was 0.9999, and the regression equation (y = 386.52x + 0.262) was 500-1.25 µg/mL. Figures 5 a, b, and c display the outcomes. It was necessary to inject each concentration three times to provide a consistent result, and the results were averaged from these three measurements (n=3). The results show that IVM and CLR both underwent linear regression with no significant non-linearities; furthermore,





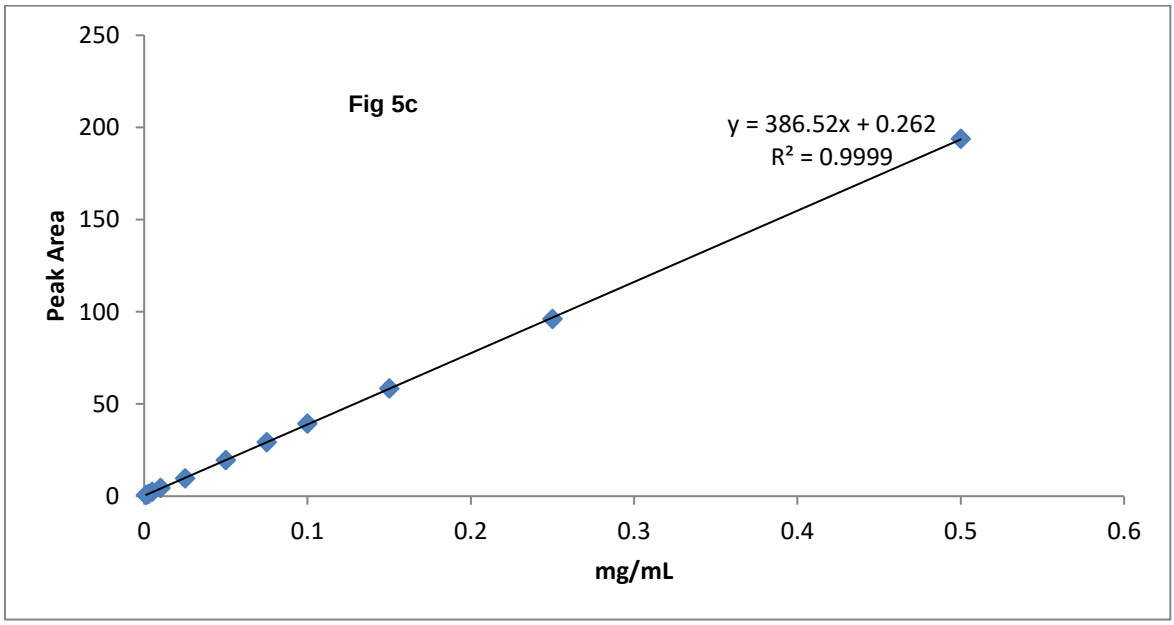


Figure 5: Calibration curve of IVMH$_2$B$_{1a}$ (a), IVMH$_2$B$_{1b}$ (b), and CLR (c) using acetonitrile: ethanol: water in the ratio of 52:28:20 (v/v) as a mobile phase with a flow rate of 1.2 mL/min at 254 nm.

3.5. Sensitivity

LOD of IVMH$_2$B$_{1a}$, IVM H$_2$B$_{1b,}$ and CLR were 0.75, 0.27, and 1.8 µg/mL, respectively. The LOQ of IVMH$_2$B$_{1a}$, IVM H$_2$B$_{1b,}$ and CLR were 2.2, 0.82, and 5.5 µg/mL, respectively. The results showed good sensitivity of the proposed method.

3.6. Precision and accuracy

As a percentage of the drug peak area response, RSD, we can measure the accuracy and precision. To determine accuracy, we analyzed samples at three distinct concentrations and, using the data from the linearity curves, computed the RSD%. The precise results for IVM H2B1a were 99.42 ± 0.43%, for IVMH2B1b they were 101.2 ± 0.05%, and for CLR they were 99.46 ± 0.66%. Tables 2, 3, and 4 provide a summary of the findings.

Table 2: Results of the accuracy of the measurements of IVM H_2B_{1a} by the proposed HPLC method. (n=3)

Theoretical Concentration in µg/mL	Found	Mean	SD	RSD%	Accuracy ± SD
47.5	48.6	48.10	0.43	0.89	101.26 ±0.43
	47.80				
	47.90				
95	93.52	93.50	0.45	0.48	98.40 ± 0.45
	33.987				
	33.62				
142.5	140.30	140.53	0.40	0.28	98.62 ± 0.40
	141.00				
	140.31				
Mean			0.43	0.55	99.42 ± 0.43
SD			0.03	0.31	1.50
RSD%					1.60

Table 3: Results of the accuracy of the measurements of IVM H_2B_{1b} by the proposed HPLC method. (n=3)

Theoretical Concentration in µg/mL	Found	Mean	SD	RSD%	Accuracy ± SD
2.5	2.50	2.52	0.03	1.20	101.80 ± 0.03
	2.50				
	2.55				
5	5.10	5.13	0.06	1.16	102.60 ± .06
	5.10				
	5.20				
7.5	7.40	7.44	0.06	0.80	99.20 ± .06
	7.45				
	7.46				
Mean			0.05	1.05	101.2± .05
SD			0.02	0.22	1.70
RSD%					1.67

Table 4: Results of the accuracy of the measurements of CLR by the proposed HPLC method (n=3).

Theoretical Concentration in µg/mL	Found	Mean	SD	RSD%	Accuracy ± SD
100	98.97	99.99	0.41	0.41	98.99 ± 0.41
	98.57				
	99.40				
150	148.36	149.32	0.89	0.60	99.55 ± 0.89
	149.51				
	150.11				
250	249.82	249.63	0.69	0.80	99.98 ± 0.69
	248.87				
	250.2				
Mean			0.66	0.60	99.46 ± 0.66
SD			0.24	0.20	0.44
RSD%					0.44

3.7. Robustness

The robustness results demonstrated that the system suitability parameters of the suggested technique were unaffected by minor variations in mobile phase pH, flow rate, and column temperature. As shown in Table 5, the robustness results for CLR, IVMH2B$_{1a}$, and IVMH2B$_{1b}$ were 0.15 mg/mL, 0.095 mg/mL, and 0.005 mg/mL, respectively.

Table 5: Results of robustness for measurements of IVM and CLR by the proposed HPLC method. (n=3).

parameter		IVM		CLR
		H_2B_{1a}	H_2B_{1b}	
Flow rate-1.1 mL/min	Retention time	13.28	11.037	2.53
	Peak Area	360.73	7.60	59.55
	Resolution	42.52	41.31	-
	Plate Count	9723	9025	8759
	Tailing factor	1.02	1.02	1.30
Flow rate-1.3 mL/min	Retention time	11.26	9.35	2.15
	Peak Area	304.75	6.24	49.53
	Resolution	39.95	38.29	-
	Plate Count	8727	8133	8298
	Tailing factor	1.02	1.02	1.26
Temperature-20°C	Retention time	13.30	10.94	2.34
	Peak Area	338.01	7.08	55
	Resolution	40.29	38.91	-
	Plate Count	8644	8100	7285
	Tailing factor	1.06	1.13	1.21
Temperature-30°C	Retention time	11.20	9.39	2.31
	Peak Area	339.93	6.99	55.11

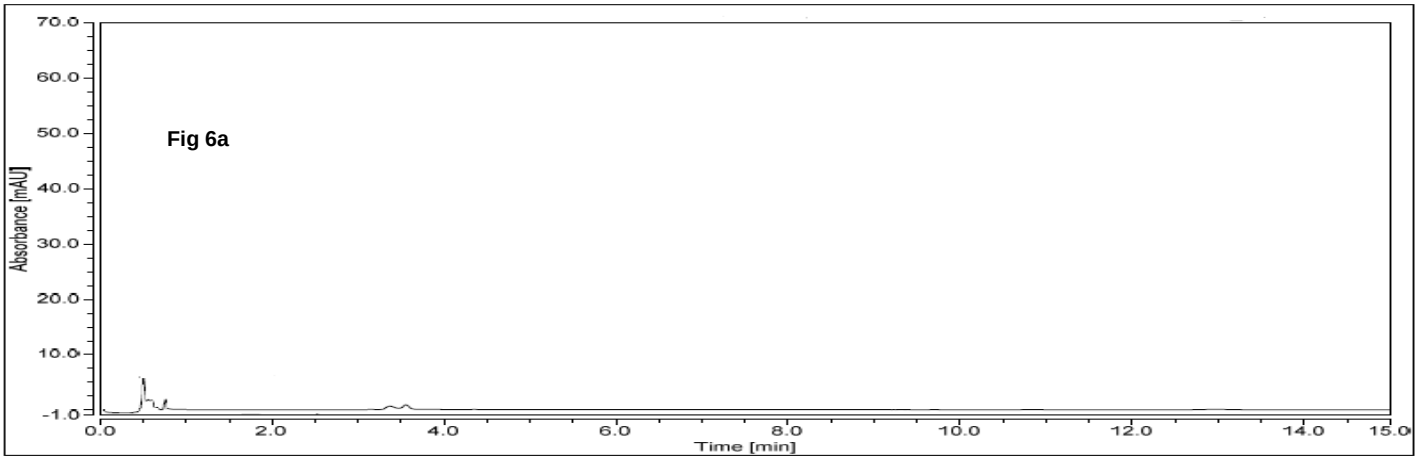
	Resolution	41.54	40.68	-
	Plate Count	9487	9191	9118
	Tailing factor	1.00	0.89	1.33
pH+2	**Retention time**	15.16	12.37	2.34
	Peak Area	342.94	6.80	56.06
	Resolution	44.73	42.24	-
	Plate Count	9033	8249	4537
	Tailing factor	1.02	0.85	1.38
pH-2	**Retention time**	12.49	10.34	2.31
	Peak Area	341.44	6.92	55.36
	Resolution	39.92	38.60	-
	Plate Count	8831	8368	8923
	Tailing factor	1.01	1.14	1.37

4. Application of the method to extract and determination of IVM and CLR in plasma goats.

A method was designed for the determination of IVM and CLR in goat plasma, utilizing the diluent solution which has the ability of precipitating proteins found in plasma and dissolving IVM and CLR that spiked in the sample. Blank plasma samples were collected from a live goat and used by the addition of drugs to the samples with different concentrations to determine the linearity, LOD, LOQ, precision, and Accuracy. Analysis of the pure filtrate directly by HPLC which the final concentration of the solution was 475, 25, and 250 µg/mL for IVM H_2b_{1a}, IVM H_2b_{1b}, and CLR, respectively. Utilizing the last sequence prepare another 3 solutions with different concentrations. For the second solution, the concentration was 356.3, 18.7, and 187.5 µg/mL for IVM H_2b_{1a}, IVM H_2b_{1b}, and CLR, respectively. For the third solution, the concentration was 237.5, 12.5, and 125 µg/mL for IVM H2b1a, IVM H2b1b, and CLR, respectively. For the fourth solution, the concentration was 118.7, 6.25, and 62.5 µg/mL for IVM H_2b_{1a}, IVM H2b1b, and CLR, respectively. Figures 6 a and b, show the chromatogram of the blank plasma and the spiked sample, respectively. This shows the ability of the method to extract and determine the two components in the matrix of plasma without any interference or overlap.

4.1. Efficiency of the method

The efficiency of the method is the ability of the method to separate and determine the spiked components in the plasma sample. The precipitating proteins should be removed and the components by dissolved in the diluent solution two times. Table 6, shows the recovery of the four solutions which were prepared in the previous step. Each drug was evaluated by 4 different concentrations triplicates (n=3). The recovery values showed the efficiency of the method.



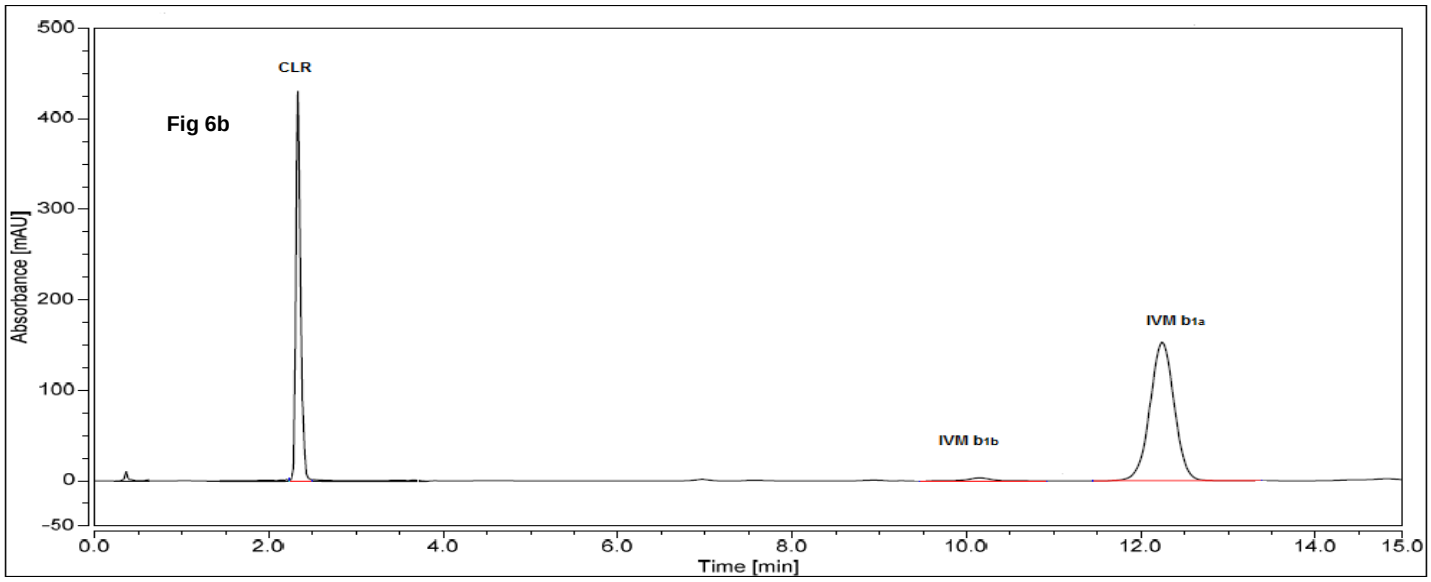


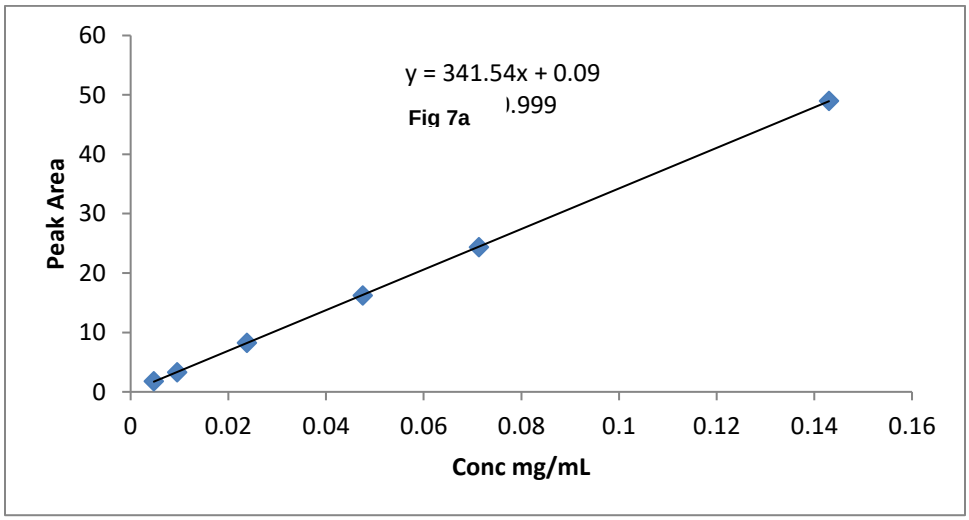
Figure 6: Chromatogram of blank plasma (a) and Chromatogram of spiked plasma sample with CLR and IVM (b) using the proposed HPLC method procedures at 254 nm.

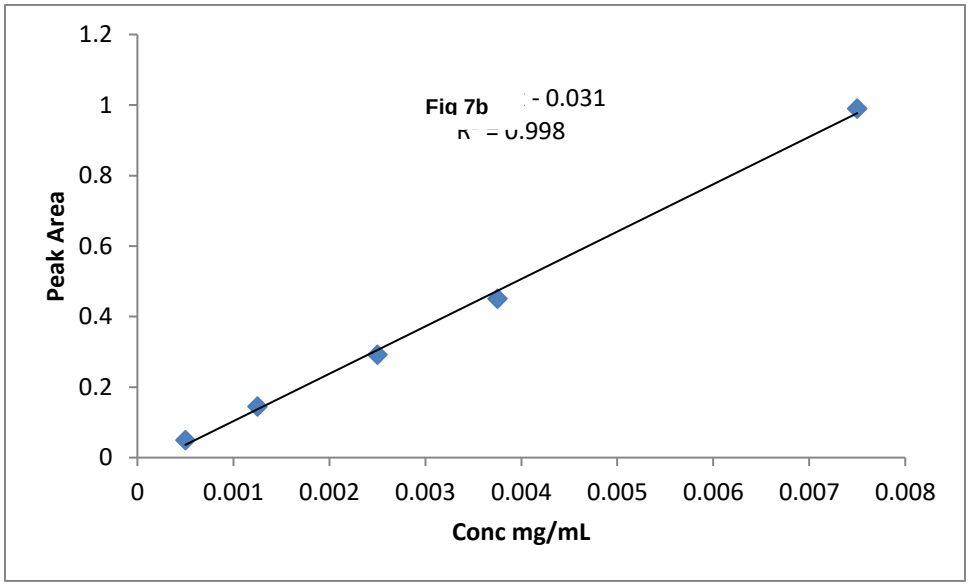
4.2. Working range and linearity

The working range was 475-2.4 µg/mL for IVMH_2B_{1a} with regression equation (y = 341.54x + 0.09) and the regression coefficient (R^2) was 0.999. Linearity was 25-0.5 µg/mL for IVMH_2B_{1b} with regression equation (y = 143. 4x - 0.031) and the regression coefficient (R^2) 0.998. Linearity was 250-2.5 µg/mL for CLR with regression equation (y = 353.36x + 0.3344) and regression coefficient (R^2) was 0.9999. Figures 7 a, b, and c show the results. Every concentration was administered three times (n=3) to ensure a consistent result, and the readings were calculated as the average of these three measurements. The results show that IVM and CLR both underwent linear regression with no significant non-linearities; furthermore.

4.3. Sensitivity

The LOD of IVMH_2B_{1a}, IVM H_2B_{1b}, and CLR were 0.85, 0.46, and 0.5 µg/mL, respectively. The LOQ of IVMH_2B_{1a}, IVM H_2B_{1b}, and CLR were 2.6, 1.41, and 1.5 µg/mL, respectively. The results demonstrate that the suggested approach is highly sensitive.





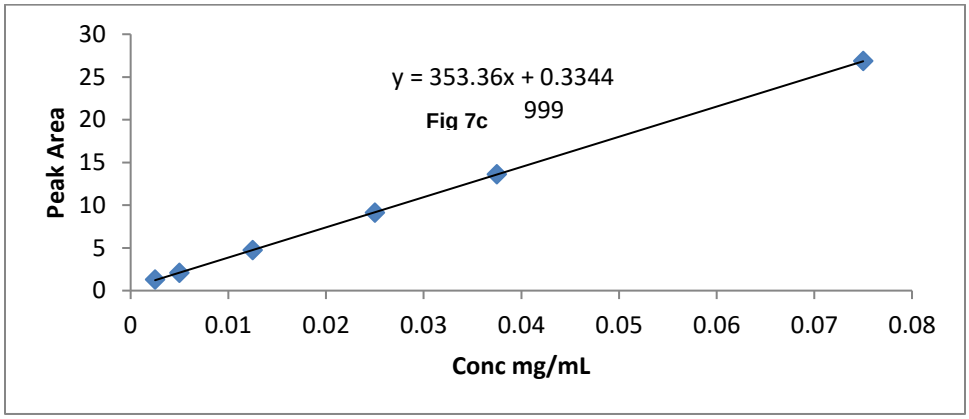


Figure 7: Calibration curve of IVMH_2B_{1a} (a), IVMH_2B_{1b} (b), and CLR (c) in plasma sample by using the proposed HPLC method at 254 nm.

4.5. Accuracy and precision

As a percentage of the drug peak area response, the intra- and inter-day measurements' precision and accuracy were computed. Tables 7 summarize the results of the calculations, which were based on analyses of samples taken at three distinct concentrations.

Table 7: Intra-day and inter-day precision and accuracy of IVM and CLR for the proposed HPLC method (n=3).

Intra-day							**Inter-day**			
Analyte		**Theoretical Conc µg/mL**	**found**	**SD**	**RSD%**	**Accuracy (RE%)±SD**	**found**	**SD**	**RSD%**	**Accuracy (RE%)±SD**
IVM	**H_2B_{1a}**	**23.8**	**23.30**	**0.15**	**0.64**	**97.89±0.15**	**23.20**	**0.12**	**0.50**	**97.50±0.12**
	H_2B_{1b}	**7.50**	**7.35**	**0.10**	**1.30**	**98.00 ± 0.1**	**7.30**	**0.10**	**1.40**	**97.30 ± 0.1**

CLR		**12.50**	**12.4**	**0.12**	**0.10**	**99.20 ± 0.12**	**12.30**	**0.20**	**1.60**	**98.40 ± 0.2**
IVM	**H_2B_{1a}**	**9.50**	**9.30**	**0.14**	**1.50**	**97.8 ± 0.14**	**9.20**	**0.12**	**1.20**	**96.80 ± 0.12**
	H_2B_{1b}	**3.75**	**3.6**	**0.08**	**2.00**	**96.00 ± 0.08**	**3.58**	**0.09**	**2.00**	**95.50 ± .09**
CLR		**5.00**	**5.00**	**0.10**	**2.00**	**100.00 ± 0.1**	**4.90**	**0.08**	**1.60**	**98.00 ± .08**
IVM	**H_2B_{1a}**	**4.75**	**4.60**	**0.10**	**2.00**	**96.80 ± 0.1**	**4.55**	**0.11**	**2.30**	**95.70 ± 0.11**
	H_2B_{1b}	**2.50**	**2.40**	**0.05**	**2.10**	**96.00 ± 0.05**	**2.40**	**0.06**	**2.50**	**96.00 ± .06**
CLR		**2.50**	**2.46**	**0.06**	**2.40**	**98.40 ± .06**	**2.40**	**0.07**	**2.90**	**96.00 ± .07**

Table 6: Efficiency of the proposed HPLC method for determination of IVM and CLR in plasma goat (n=3).

5. Conclusion

IVM H_2B_{1a}								IVM H_2B_{1b}							CLR						
Theoretical Conc µg/mL	found	Mean	SD	REC %	RSD %	REC ± SD	Theoretical Conc µg/mL	found	Mean	SD	REC %	RSD %	REC ± SD	Theoretical Conc µg/mL	found	Mean	SD	REC %	RSD %	REC ± SD
475	466.63	466.8	0.39	98.27	0.1	98.27± 0.39	25	24.68	24.72	0.09	98.88	0.36	98.88± 0.09	250	251.12	251.26	0.17	100.50	0.07	100.5±0.17
	467.25							24.82							251.45					
	466.52							24.66							251.21					
356.3	349.97	349.7	0.32	98.09	0.1	98.09± 0.32	18.7	18.43	18.18	0.41	97.24	2	97.24± 0.41	187.5	188.3	188.59	0.33	100.58	0.17	100.58±0.33
	349.8							17.71							188.95					
	349.35							18.41							188.53					
237.5	231.35	231.8	0.45	97.58	0.2	97.58± 0..45	12.5	12.15	12.14	0.04	97.12	0.33	97.12± 0.04	125	124.64	124.72	0.14	99.78	0.11	99.78±0.14
	231.69							12.1							124.64					
	232.25							12.17							124.89					
118.7	116.62	116.3	0.45	97.96	0.38	97.96± 0.45	6.3	6.1	6.15	0.05	97.57	0.81	97.57± 0.05	62.5	62.96	62.63	0.28	100.21	0.45	100.21±0.28
	115.87							6.14							62.48					
	116.36							6.2							62.46					
Mean			0.41	97.98	0.2	97.98±0 .41				0.15	97.70	0.88	97.70± 0.15				0.23	100.27	0.2	100.27±0.23
SD			0.06	0.29	0.12					0.18	0.81	0.78					0.09	0.36	0.17	
RSD%				0.30							0.83							0.36		

We created a chromatographic method to efficiently determine IVM and CLR that is simple, quick, accurate, isocratic, and reproducible. The need for chemicals and organic solvents helped to clarify how easy the procedure was to implement. The proposed method was found to be linear, sensitive, selective, specific, precise, accurate, and robust based on the results of the method validation experiments. It also exhibited stability-indicating characteristics, so quality control laboratories can use it for routine quality control of IVM and CLR. Comparing our new method to previously reported methods, as shown in Table 8, reveals that the former has better linear range limits, LOD, and LOQ, as well as better peak analysis parameters such as plate count and tailing factor.

Table 8: Comparison between the new method and the reported method

Method No.	compound	Linear Range µg/mL	R^2	RSD%	LOD µg/mL	LOQ µg/mL	Accuracy%	Tailing factor	Plate	Reference No.
1	IVM	10-40	0.9998	0.26	0.61	1.80	99.1-99.7	1.92	1794	2
2		30-300	0.9995	1.8	6.15	20.50	100-100.1	1.43	1880	5
3		2.5-7.5	0.9999	1.65	0.06	0.20	100.34	1.38	7222	6
4		0.001-1.5 mg/mL	0.999	1.7	1	0.3	99.2-100.2	-	-	11
New method	H_2B_{1a}	950-3.8	0.999	1.64	0.75	2.2	98.5-101.2	0.96	9604	Present method
	H_2B_{1b}	50-0.5	0.9999	1.1	0.27	0.82	99.1-101.2	0.81	9473	
1	CLR	100-400 L	0.9998	1.11	6.16	18.68	98.7-99.7	0.97	5557	2
2		0.625-25	0.9999	1.3	0.16	0.54	99.2-99.9	1.12	1735	5
3		25-75 µg/mL	0.9999	0.1	0.61	1.86	99.76	1.53	5785	6
4		0.001-1.5 mg/mL	0.999	0.7	1	0.3	99.1-100.6	-	-	11
New method		500-1.25	0.9999	0.5	1.8	5.5	98.8-99.8	1.2	8140	Present method

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