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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF IVERMECTIN AND CLORSULON IN IVERCAM INJECTION

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ABSTRACT

A precise, simple, accurate and selective method was developed and validate for estimation of Ivermectin and Clorsulon in Ivercam injection, Reversed phase high performance liquid chromatographic (RP-HPLC) method was developed for routine quantification of Ivermectin and Clorsulon in laboratory prepared mixtures as well as in combined dosage form. Chromatographic separation was achieved on a BDS hypersil C_{18} (5µ, 250 x 4.6 mm) utilizing mobile phase of filtered and degassed mixture of 60 phosphate buffer (pH 5.5 adjusted with 1% O-phosphoric acid) and Methanol (60:40 v/v) at a flow rate of 1 mL/min with UV detection at 234 nm. The method has been validated for linearity, accuracy and precision. In RP-HPLC method, the calibration graphs were linear in the concentration range of 2.5-7.5 µg/ml for Ivermectin and 25-75 µg/ml for Clorsulon with percentage recoveries of 100.34 % and 99.76% for Ivermectin and Clorsulon respectively. Conclusion: The results obtained by RP-HPLC methods are rapid, accurate and precise. Therefore proposed method can be used for routine analysis of Clorsulon and Ivermectin in injection.

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INTRODUCTION

Ivermectin (IVR) is chemically 22, 23-dihydroavermectin B1a+ 22, 23-dihydroavermectin B1b (**Figure: 1**); is an extremely potent semisynthetic derivative of an antinematodal principle obtained from *Streptomyces avermitilis*. It binds to glutamate-gated chloride ion channels which occur in invertebrate nerve and muscle cells. This leads to an increase in the permeability of the cell membrane to chloride ions with hyper polarization of the nerve or muscle cell, resulting in paralysis and death of the parasite [1-5].

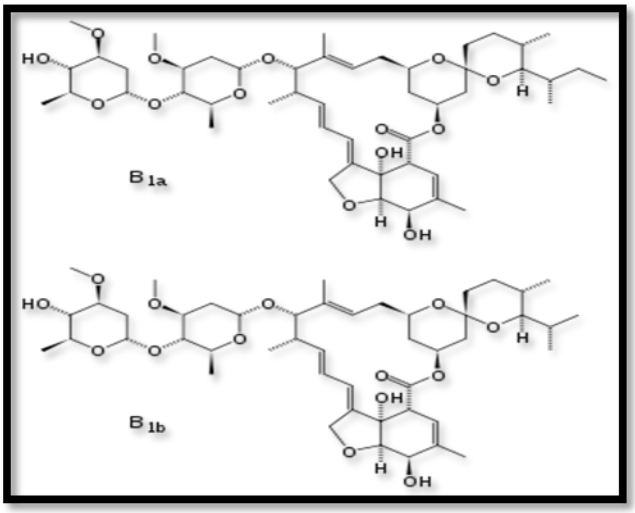

Figure: 1 Structure of Ivermectin.

Clorsulon (CLOR) is 4-amino-6-(1,2,2-trichloroethenyl)-benzene-1,3-disulfonamide **(Figure: 2**); this drug has good efficacy against mature rather than immature flukes when is given orally or via subcutaneous injection [3, 4]. Clorsulon is specific against Fasciola spp. used for cattle at dose of 7 mg/kg to effect at great extent against fluke aged 8 weeks or older but the suggested (not registered) dose rate against immature flukes for sheep is 15 mg/kg [6-7].

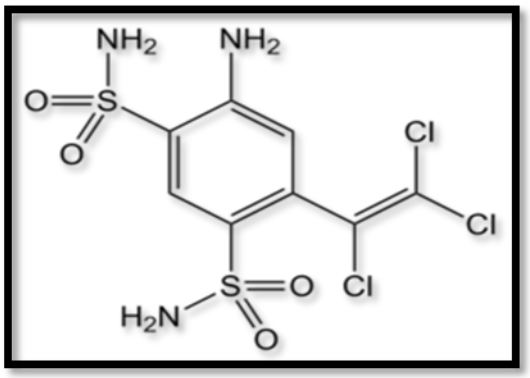

Figure: 2 Structure of Clorsulon.

Uses of dosage form

Used in effective treatment and control of internal parasites, including adult liver flukes, and external parasites in cattle.

Objective:

Development and validation of High performance liquid chromatography method for estimation of Ivermectin and Clorsulon in injection .

Introduction to dosage form

IVERCAM injection was manufactured by Kamdhenu Stermed (Ahmedabad). Each injction claimed to contain 10mg/ml of Ivermectin and 100mg/ml of Clorsulon.

MATERIAL AND METHODS

Reagent and Chemicals:

Pure Samples

Clorsulon and Ivermectin working standard was kindly gifted by NGL Fine Chem ,Mumbai.

Chemicals used in RP-HPLC method

Acetonitrile (HPLC), Potassium dihydrogen phosphate, Water (HPLC), - Orthophosphoric acid (AR), Triethylamine (AR) and Methanol (HPLC) were procured from Merck, Rankem.

System suitability parameters

System suitability parameters like average peak area of standards, resolution, tailing factors etc. has been measured and data were shown in Table 1.

Table 1. System suitability parameters.

System Suitability Parameters	Clorsulon	Ivermectin
No. of theoretical plates	5785	7222
Retention Time (min)	3.770	5.657
Tailing factor	1.53 ± 0.016	1.38 ± 0.012
Rsolution	8.123	

Wavelength determination

The detection was carried out in the UV region and wavelength selected for detection was 234 nm in mobile phase. 10µg/ml of Clorsulon and Ivermectin working standard solutions were separately prepared in methanol. The zero order overlain spectrums of the prepared solutions were recorded from 200 to 400 nm in double beam UV-visible spectrophotometer (Shimadzu, model 1800). The isoabsorptive point was found at 234 nm and it was selected as wavelength of determination for both the drugs. The overlain spectrum of Clorsulon and Ivermectin was shown in Figure: 3.

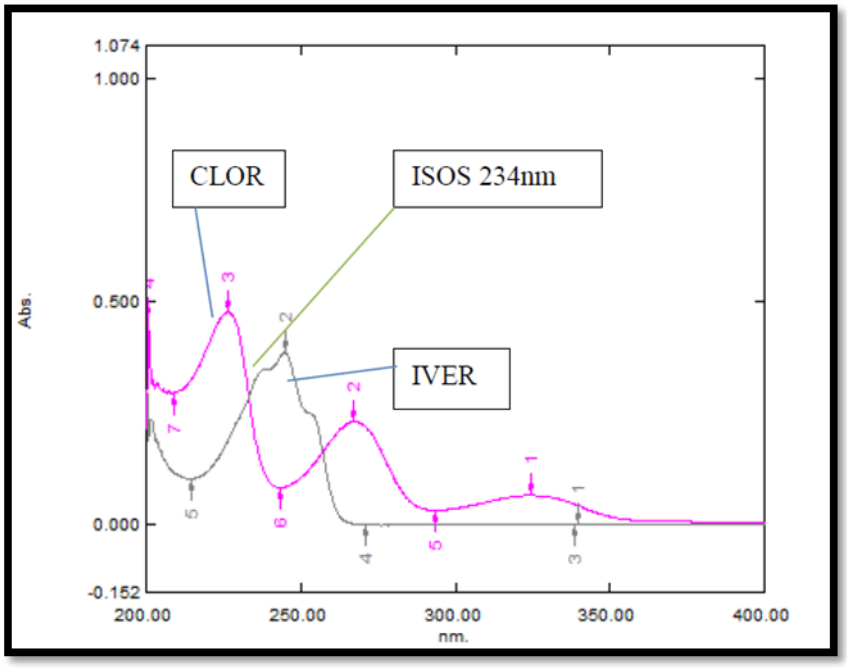

Figure: 3 Overlain UV spectra of CLOR (10µg/ml) and IVER (10µg/ml).

Chromatographic conditions

The HPLC system consisted of Shimadzu LC-20A system equipped with model LC-20AT pump, SPD- 20AT Shimadzu UV-Vis (Diode array) detector, Hamilton syringe and DGU-20A5 online degasser, and a Rheodyne injection valve. Peak areas were integrated using a Spinchrom Software program. Experimental conditions were optimized on a BDS Hypersil C 18 column (5µ, 250 x 4.6 mm), Thermo scientific at room temperature using 0.1 % KH_2PO_4: Methanol (60:40V/V) as mobile phase. Mobile phase was flowed at 1 mL/min and all chromatographic experiments were performed at room temperature (25 ˚C ± 2˚C).

Preparation of mobile phase

Preparation of KH_2PO_4:

2.72 gm of phosphate buffer was accurately weighed and dissolved in 1000ml of water, and then pH 5.5 was adjusted using 1% O-phosphoric acid.

Mobile Phase: Prepare a filtered and degassed mixture of 60 volumes of buffer preparation (pH 5.5) and 40 volumes of Methanol.

Preparation Standard of stock solution

a) Stock solution of Ivermectin

Standard stock solution of Ivermectin was prepared by dissolving 10 mg of drug with mobile phase in 10 ml of volumetric flask and made up to volume to get concentration of 1000 µg/ml

b) Stock solution of Clorsulon

Standard stock solution of Clorsulon was prepared by dissolving 10 mg of drug with mobile phase in 10 ml of volumetric flask and made up to volume to get concentration of 1000 µg/ml.

Preparation of Sample solution

1 ml solution containing 10mg Ivermectin and 100mg Clorsulon from formulation was taken and dissolved in mobile phase and made upto 10ml. So, the concentration of Ivermectin was 1000µg/ml and Clorsulon was 10,000 µg/ml. It was further diluted to 100µg/ml and 1000µg/ml by taking 1ml from above solution and diluting it upto
10ml.Further dilutions were made according to the requirement.

Standard Solutions for linearity

Linearity was studied by preparing standard solutions at 5 different concentrations. The linearity range for Clorsulon and Ivermectin were found to be 25-75µg/ml and 2.5-7.5µg/ml, respectively. Calibration curve were obtained by plotting respective peak area against concentration in µg/mL and the regression equation was computed.

Validation of the developed method:

Validation of the developed method done using following parameters as per International Conference on Harmonization (ICH Guidelines) [8-10].

1.Specificity
2. Linearity
3.Range
4.Accuracy
5.Precision
6.Detection limit
7.Quantitation limit
8.Robustness
9.System suitability testing

Method validation [11]**:**

Linearity and range:

The linearity response was determined by analyzing 5 independent levels of calibration curve in the range of 25-75µg/ml and 2.5-7.5µg/ml for CLOR and IVER respectively. Plot the calibration curve of Area versus respective concentration and find out correlation co-efficient and regression line equation for CLOR and IVER.

Precision

a) Intra-day precision

For Intraday precision, it was carried out by preparing 3 replicates of 3 different concentrations, within the linearity range and measuring the peak area of each solution on the same day. % RSD (% relative standard deviation) was calculated.

b) Inter-day precision

For Interday precision, it was carried out by preparing 3 replicates of 3 different concentrations, within the linearity range and measuring the peak area of each solution on 3 different days. % RSD (% relative standard deviation) was calculated.

Accuracy

To a fixed amount of pre-analyzed sample of CLOR 25µg/ml and IVER 2.5µg/ml, increasing amount working standard solution of CLOR (20 ,25 and 30 ug/ml) and IVER (2, 2.5, and 3µg/ml) were added in 10 ml volumetric flask and made up to mark with mobile phase Samples were injected into system and analyzed as described. Calculate the mean % recovery from peak areas obtained.

Limit of Detection (L.O.D.)

The L.O.D. was estimated from the set of 5 calibration curves used to determine method linearity. The L.O.D. may be calculated as

$$\mathbf{LOD = 3.3 \times (\sigma/S)}$$

Where,

σ = Standard deviation of the Y- intercepts of the 5 calibration curves.

S = Mean slope of the 5 calibration curves.

Limit of Quantification (L.O.Q.)

The L.O.Q. was estimated from the set of 5 calibration curves used to determine method linearity. The L.O.Q. may be calculated as

$$LOQ = 10 \times (\sigma/S)$$

Where,

σ = Standard deviation of the Y-intercepts of the 5 calibration curves.

S = the mean slope of the 5 calibration curves.

Robustness

For robustness evaluation of HPLC method a few parameters like flow rate, mobile phase composition and pH were deliberately changed. Robustness of the method was done at the concentration level 50 µg/ml and 5 µg/ml for CLOR and IVER respectively.

RESULT AND DISCUSSION

Linearity and Range

The linearity range for CLOR was found to be in the range of 25-75µg/ml and for IVER it was 2.5-7.5 µg/ml. Calibration data for CLOR and IVER is presented in Table 2 and Regression analysis obtained are presented in Table 3. Calibration curve of Clorsulon shown in Figure: 4 and Calibration curve of Ivermectin Shown in figure:5.

Table 2: Calibration data for Clorsulon and Ivermectin.

CLOR (µg/ml)	IVER (µg/ml)	Peak area Avg. area (n=5)	
		CLOR	IVER
25	2.5	930.27	607.125
32.5	3.25	1394.17	909.35
50	5	1882.14	1229.92
62.5	6.25	2340.86	1529.93
75	7.5	2816.65	1840.54

Table 3: Regression analysis for Clorsulon and Ivermectin.

Regression analysis	CLOR	IVER
Correlation coefficient	0.9999	0.9999
Slope	37.75	246.99
Intercept	14.95	11.59

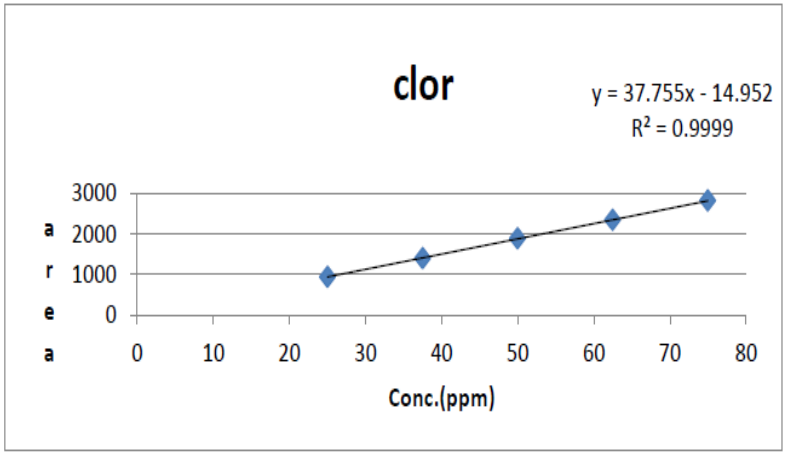

Figure 3: Calibration curve of Clorsulon.

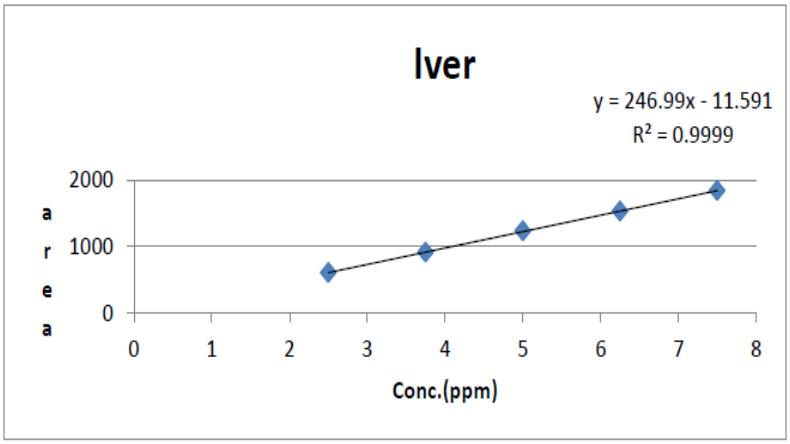

Figure 4: Calibration curve of Ivermectin.

Precision

Table: 4. Repeatability for Clorsulon and Ivermectin.

Drug	Average	SD	RSD
Clorsulon	1879.46	1.99	0.10
Ivermectin	1237.28	20.52	1.65

Intra-day precision

The data for intraday precision of CLOR and IVER are presented in Table 5 and Table 6 respectively. The % R.S.D. for Intra-day precision was found to be 0.11-0.15 % for CLOR and 0.201-0.203% for IVER.

Table: 5. Intraday precision for Clorsulon:

	Conc.µg/ml	Avg. area	Standard deviation	%RSD
50%	25	923.16	1.38	0.15
100%	50	1869.64	2.22	0.11
150	75	2796.60	3.36	0.12

Table: 6. Intraday precision for Ivermectin.

	Conc. µg/ml	Avg. area	Standard deviation	%RSD
50%	2.5	603.46	1.22	0.20
100%	5.0	1222.54	2.46	0.20
150	7.5	1829.48	3.71	0.20

Inter-day precision

The data for inter-day precision of CLOR and IVER are summarized in Table 7 and Table 8 respectively. The % R.S.D. for inter-day precision was found to be 0.100-0.106 % CLOR and 0.19-0.20 % for IVER.

Table 7. Interday precision for Clorsulon.

	Conc. µg/ml	Avg. area	Standard deviation	%RSD
50%	25	924.52	0.98	0.10
100%	50	1870.63	1.91	0.10
150	75	2799.88	2.81	0.10

Table 8. Interday precision for Ivermectin.

	Conc. µg/ml	Avg. area	Standard deviation	%RSD
50%	2.5	604.08	1.19	0.19
100%	5.0	1223.78	2.45	0.20
150	7.5	1831.33	3.62	0.19

Accuracy

Accuracy of the method was confirmed by recovery study from marketed formulation at three level of standard addition. Percentage recovery for CLOR was 98.9-101.3%, while for IVER, it was found to be in range of 99.3-101.5%, The results are shown in Table. 9 and Table. 10. Recovery greater than 98 % justifies the accuracy of the method.

Table 9 Recovery data for Clorsulon.

	Amt of sample (µg/ml)	Amt of std. spiked(µg/ml)	Avg.Amt. recovery	%recovery ± SD	%RSD
80%	25	20	19.96	99.82 ± 1.3	1.32
100%	25	25	24.93	99.73 ± 0.8	0.82
120%	25	30	29.91	99.72 ± 0.6	0.64

Table 10 Recovery data for Ivermectin.

	Amt of sample (µg/ml)	Amt of std. spiked(µg/ml)	Avg.Amt.recovery	%recovery ± SD	%RSD
80%	2.5	2	2.009	100.59 ± 1.12	1.12
100%	2.5	2.5	2.50	100.24 ± 0.65	0.65
120%	2.5	3	3.005	100.19 ± 0.49	0.49

Limit of Detection & Limit of Quantification

The LOD and LOQ value for CLOR and IVER are shown in Table 11.

Table 11. LOD and LOQ for Clorsulon and Ivermectin.

	Clorsulon	Ivermectin
LOD	0.61	0.06
LOQ	1.86	0.20

Robustness

The data for robustness are presented in Table 12 and 13.

Table 12. Robustness for Clorsulon.

Standard	Variation	Area	%RSD
pH	+0.2	1795.14	0.34
	-0.2	1932.94	0.39
Mobile phase	+2	1836.52	0.34
	-2	1931.88	0.33
Flow rate	+0.2	1836.68	0.45
	-0.2	1951.61	0.37

Table 13. Robustness for Ivermectin.

Standard	Variation	Area	%RSD
pH	+0.2	1174.27	0.62
	-0.2	1264.58	0.58
Mobile phase	+2	1201.20	0.61
	-2	1264.58	0.58
Flow rate	+0.2	1200.00	0.61
	-0.2	1276.90	0.58

Applicability of the Method

Applicability of the proposed method was tested by analyzing the commercially available injection formulation. The results are shown in Table 14.

Table 14. Analysis of Marketed Formulation.

	Sr.no	Label claim (mg)	Result (w/w)	%Assay	Avg %Assay	SD	%RSD
Clorsulon 50µg/ml	1	100	99.91	99.91			
	2	100	100.11	100.11	99.97	0.12	0.12
	3	100	99.88	99.88			
Ivermectin 5µg/ml	1	10	9.99	99.91			
	2	10	10.01	100.11	100.11	0.19	0.19
	3	10	10.03	100.31			

Summary of Parameters

Table 15. Summary of parameters.

Parameters		Result	
		CLOR	IVER
Linearity		0.999	0.999
Range		25-75 µg/ml	2.5-7.5 µg/ml
Accuracy	80%	99.82 ± 1.3	100.59 ± 1.12
	100%	99.73 ± 0.8	100.24 ± 0.65
	120%	99.72 ± 0.6	100.19 ± 0.49
Precision	Inter day	%RSD=0.100-0.106	%RSD=0.19-0.20
	Intra day	%RSD=0.11-0.15	%RSD=0.201-0.203
	Inter day	%RSD=0.1059	%RSD=1.6589
LOD		0.61 µg/ml	0.06 µg/ml
LOQ		1.86 µg/ml	0.20 µg/ml

CONCLUSION

The proposed RP-HPLC method was accurate and precise and applicable for the determination of Ivermectin and Clorsulon without interference and with good sensitivity. Therefore proposed method can be used for routine analysis of Clorsulon and Ivermectin in injection.

Recommended future work: Stability indicating study

Abbreviations

HPLC : High Performance Liquid Chromatography
UV : Ultra violet
Mm : Millimetre
µm : Micrometre
LOD : Limit of Detection
LOQ : Limit of Quantitation
ICH : International Conference on Harmonisation
mL : Millilitre
µg/mL : Microgram/Millilitre
r^2 : Correlation coefficient
mg : Milligram
kg : Kilogram
% : Percentage
% RSD : Percent relative standard deviation
CLOR : Clorsulon
IVER : Ivermectin

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Conflict of interest:
There is a no conflict of interest

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