Development and Validation for HPLC Method of Assay of Ivermectin and Clorsulon in Combined Pharmaceutical Dosage Form

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Abstract: Stability indicating-HPLC method has been developed for simultaneous estimation of Ivermectin and Clorsulon in their combined dosage form. For RP-HPLC method, all the standard and sample solutions were prepared in methanol. A RP-HPLC method has been developed and subsequently validated for simultaneous estimation of Ivermectin and Clorsulon in their combination product. The proposed RP-HPLC method utilizes a Thermo BDS C-18 (15cm x 4.6mm, 5 μ m) column, mobile phase consisting of acetonitrile, methanol and purified water in the proportion of 60: 30:10 (v/v/v), and UV detection at 245 nm. The described method was linear over a range of 10-40 μ g/ml with a correlation coefficient (r^2) of 0.9998 for Ivermectin and a range of 100-400 μ g/ml with a correlation coefficient (r^2) of 0.9998 for Clorsulon. Validations of the proposed method were carried out for its accuracy, precision, linearity and range, specificity, LOD and LOQ according to ICH guidelines. A stability-indicating study was also carried out and indicated that this method can also be used for purity and degradation evaluation of these formulations that occurred due to temperature, humidity and time. the method has been successfully applied for the analysis of drugs in formulation.

Keywords: Ivermectin, Clorsulon, RP-HPLC, Limit of Detection, Limit of Quantitation

1. Introduction

Ivermectin (IVM) is macrocyclic lactone that has been known as a potent, effective and safe antiparasitic drug for 20 years [1]. It is widely used as an antiparasitic agent in domestic animals and is considered the drug of choice for lymphatic filariasis and river blindness (onchocerciasis) in humans [2]. IVM is a member of the Avermectins; this group includes natural compounds produced by fermentation of the soil-dwelling actinomycete *Streptomyces avermitilis*. IVM, a semi-synthetic derivative of avermectin B1, consists of an 80:20 mixtures of the equipotent homologous 22, 23 dehydro B1a and B1b [3].

Clorsulon (CLO) is an antihelminthicum. It is used against the adult forms of parasitic flatworms in cattle, in particular from the liver fluke *Fasciola hepatica*, and against *Fasciola gigantica* [4]. Clorsulon is chemically 4-Amino-6-(trichlorovinyl) benzen-1, 3-disulfonamide [5].

Literature survey reveals a few spectrophotometric and chromatographic methods for the estimation of both drugs as a single component and in combination with other drug [6]. However, no method has been reported for analysis of these drugs in combined dosage form. there is official method for simultaneous estimation of the two drugs in their combined form stated in, United states [7], British [8] or European Pharmacopeias [9].

Ivermectin is a semisynthetic, anthelmintic agent for oral administration. Ivermectin is derived from the avermectins, a class highly active broad-spectrum, anti-parasitic agents isolated from the fermentation products of *Streptomyces avermitilis*. Ivermectin is a mixture containing at least 90% 5-O-demethyl-22,23-dihydroavermectin A1a and less than 10% 5-O-demethyl-25-de (1-methylpropyl)-22,23-dihydro-25-(1-methylethyl) avermectin A1a, generally referred to as 22,23-dihydroavermectin B1a band B1b, or H2B1a and H2B1b,

respectively. The respective empirical formulas are $C_{48}H_{74}O_{14}$ and $C_{47}H_{72}O_{14}$, with molecular weights of 875.10 and 861.07, respectively. The structural formulas are: component B1a, $R \equiv C_2H_5$ component B1b, $R \equiv CH_3$ (see figure 1).

Ivermectin is a white to yellowish-white, nonhygroscopic, crystalline powder with a melting point of about $155^\circ C$. It is insoluble in water but is freely soluble in methanol and soluble in 95% ethanol [10].

Figure 1. Below is Ivermectin structural formula.

On livestock ivermectin is effective against the major parasitic roundworms: gastrointestinal (e.g. *Haemonchus* spp, *Cooperia* spp, *Ostertagia* spp and *Trichostrongylus* spp) and pulmonary (e.g. *Dictyocaulus* spp). It is also effective against most mites and lice species, and against numerous myiasis (e.g. those caused by screwworm flies, bot flies and warble flies) usually regardless of the delivery form (pour-on, injectable, drench or feed additive).

Clorsulon is a substance belonging to the benzenesulphonamide family which is used for the treatment and control of adult flukes. Veterinary medicinal products containing clorsulon are currently marketed in the EU for the treatment of cattle. They are available as injectable formulations to be administered subcutaneously (recommended dose 2 mg/kg) or by the oral route (recommended dose 7 mg/kg). Clorsulon is frequently used in association with Ivermectin [11].

Figure 2. Below is Clorsulon structural formula.

Clorsulon is frequently used in association with Ivermectin. Clorsulon was previously assessed by the CVMP and a toxicological ADI of 0.002 mg/kg body weight, i.e. 0.120 mg/person was established.

In susceptible flukes, Clorsulon inhibits the glycolytic enzymes 3-phosphoglycerate kinase and phosphoglyceromutase, thereby blocking the Emden-Myerhof glycolytic pathway. The fluke is deprived of its main metabolic energy source and dies.

Clorsulon is approved for use in the treatment of immature

and adult forms of *Fasciola hepatica* (Liver fluke) in cattle. It is not effective against immature flukes less than 8 weeks old. It also has activity against *Fasciola gigantica*. Although not approved, the drug has been used in practice in various other species (e.g., sheep, llamas). It has activity against *F. magna* in sheep, but is not completely effective in eradicating the organism after a single dose, thereby severely limiting its clinical usefulness against this parasite. Clorsulon is also not effective against the rumen fluke (*Paramphistomum*). Therefore, the objective of present communication is to develop simple, rapid and precise spectrophotometric method for the estimation of Ivermectin and clorsulon in combined pharmaceutical dosage form.

2. Experimental

Instruments and reagents

HPLC Model (Shimadzu, Kyoto, JAPAN), Auto sampler (SIL-20AC), UV/VIS Detector (YL9120), SPD-20AV, prominence Liquid Chromatography model LC-20AB, prominence column Oven model CTO-20A, Degassing Unit model DGU-20A3R & L C solution software is used for Data acquisition and analysis.

Ivermectin Working Standard (96.2% purity) was obtained from Aurum Research Centre (Amman, Jordan), Clorsulon Working Standard (100% purity) was obtained from India Pharma, Acetonitrile (HPLC Grade) & Methanol (HPLC Grade) were used is obtained from CARLO ERBA Reagents (Italy). The $0.45\mu m$ nylon filters were purchased from VIVID Separation & Filtration (Amman, Jordan), Ivermectin plus Injection (Ivermectin & Clorsulon) Samples Batch No (T-05) used in Validation are granted by Bash Pharma Pharmaceutical and all other facilities.

Liquid Chromatographic Conditions

Chromatographic conditions were obtained using a stainless steel column (Thermo BDS C-18, 150mm x 4.6mm $5.0\mu m$), which was maintained at ambient temperature. The analytical wavelength was set at 245 nm and samples of 10 μl were injected to HPLC system. The mobile phase consisting of a mixture of Acetonitrile: Methanol: Purified Water in the ratio (60:30:10), at a flow rate of 1ml/min. The mobile phase was filtered through $0.45\mu m$ filter and degassed for 10 minutes by sonication.

Standard Solutions

Standard Stock Solutions

An accurately weighed quantity of 20 mg of Ivermectin and 200 mg of Clorsulon were transferred into a 100 ml volumetric flask. Methanol was added to mark to produce a solution having a concentration of 200 $\mu g/ml$ of Ivermectin and 2000 $\mu g/ml$ of Clorsulon.

Preparation of Working Standard

From the standard stock solution 5 ml is pipette out into 50 ml volumetric flask and made up the volume with methanol to produce a solution having a concentration of 20 $\mu g/ml$ of Ivermectin and 200 $\mu g/ml$ of Clorsulon.

Preparation of Assay Working Solution

2 ml of Ivermectin plus (clorsulon 100 mg + Ivermectin 10

mg) equivalent to 200mg and 20 mg of Clorsulon and Ivermectin respectively is transferred into a 100 ml volumetric flask. Methanol is added to the mark and ultrasonicated for 5 minutes to produce a solution having a concentration of 200 µg/ml of Ivermectin and 2000 µg/ml of Clorsulon.

5 ml of this solution is diluted to 50 ml with the same solvent to produce a Solution having the concentration of 20 µg/ml of Ivermectin and 200 µg/ml of Clorsulon.

Method Validation

Method validation is the process of proving that an analytical method is acceptable for its intended purpose [12]. For pharmaceutical methods, guidelines from the United States Pharmacopeia (USP), International Conference on Harmonization (ICH), and the Food and Drug Administration (FDA) provide a framework for performing such validations. In general, methods for regulatory submission must include studies on specificity, linearity, accuracy, precision, range, detection limit, quantitation limit, and robustness [13].

Procedure

System Suitability Test

From the standard working solution, the concentration was prepared having 20 µg/ml of Ivermectin and 200 µg/ml of Clorsulon. The system suitability test was performed from Five replicate injections of standard working solution.

Linearity

In order to study linearity of the response, a series of Seven concentrating levels from 50% - 200% of assay analytes concentrations were prepared from stock solutions. The linearity performed was used for the determination of limits of quantification and detection.

From standard stock solution having the concentration 200 µg/ml of Ivermectin and 2000 µg/ml of Clorsulon.

Transfer accurately the specified volume into each of 100-ml volumetric flask to obtain the required working standard concentrations explained as follows:

Clorsulon Linearity

100 µg /ml: 5 ml was transferred from 2000 µg/ml standard stock solution to 100-ml volumetric flask, then made up to volume with Methanol (50%).

150 µg /ml: 7.5 ml was transferred from 2000 µg/ml standard stock solution to 100-ml volumetric flask, then made up to volume with Methanol (75%).

200 µg /ml: 10 ml was transferred from 2000 µg/ml standard stock solution to 100-ml volumetric flask, then made up to volume with Methanol (100%).

250 µg /ml: 12.5 ml was transferred from 2000 µg/ml standard stock solution to 100-ml volumetric flask, then made up to volume with Methanol (125%).

300 µg /ml: 15 ml was transferred from 2000 µg/ml standard stock solution to 100-ml volumetric flask, then made up to volume with Methanol (150%).

350 µg /ml: 17.5 ml was transferred from 2000 µg/ml standard stock solution to 100-ml volumetric flask, then made up to volume with Methanol (175%).

400 µg /ml: 20 ml was transferred from 2000 µg/ml standard stock solution to 100-ml volumetric flask, then made up to volume with Methanol (200%).

Ivermectin Linearity

10 µg /ml: 5 ml was transferred from 200 µg/ml standard stock solution to 100-ml volumetric flask, then made up to volume with Methanol (50%).

15 µg /ml: 7.5 ml was transferred from 200 µg/ml standard stock solution to 100-ml volumetric flask, then made up to volume with Methanol (75%).

20 µg /ml: 10 ml was transferred from 200 µg/ml standard stock solution to 100-ml volumetric flask, then made up to volume with Methanol (100%).

25 µg /ml: 12.5 ml was transferred from 200 µg/ml standard stock solution to 100-ml volumetric flask, then made up to volume with Methanol (125%).

30 µg /ml: 15 ml was transferred from 200 µg/ml standard stock solution to 100-ml volumetric flask, then made up to volume with Methanol (150%).

35 µg /ml: 17.5 ml was transferred from 200 µg/ml standard stock solution to 100-ml volumetric flask, then made up to volume with Methanol (175%).

40 µg /ml: 20 ml was transferred from 200 µg/ml standard stock solution to 100-ml volumetric flask, then made up to volume with Methanol (200%).

Statistical Analysis and Calculation Formula Used

Linearity data should be evaluated using appropriate statistical methods.

A simple regression line of the detector response versus the sample concentration is the most common means of evaluation. Regulatory agencies require the submission of the correlation coefficient, y-intercept, slope of the regression line, and the residual sum of squares for linearity evaluation.

For the current study statistics data analysis (Stata Software) is used (Texas, USA).

Formula:

$$\text{Assay\%} = \frac{A_t}{A_s} \times \frac{C_s}{C_t} \times 100$$

Where: A_t = Area of the Test

A_s = Area of the Standard

C_s = Concentration of the Standard

C_t = Concentration of the Test

Limit of Detection

The LOD is the smallest amount of analyte that can be detected, but not necessarily quantitated using a given method. This parameter is important in the use of limit tests as it sets the level below which the method cannot function.

There are several means of calculating LOD for HPLC methods. The most common approach is to determine the sample amount that provides a signal-to-noise ratio of 3:1. Many chromatographic data acquisition systems provide integrated functions to determine the signal-to-noise ratio that are easily employed by analyst.

An alternative to the signal-to-noise approach is to estimate LOD based on the standard deviation of response. For this calculation, $\text{LOD} = 3.3(\text{SD}/S)$, where SD is the standard deviation of the response based on the standard deviation of the blank, the residual standard deviation of the regression line, and the standard deviation of the y-intercepts of the regression

line, and S is the slope of the calibration curve.

Root MSE (SD) = the standard deviation of the y-intercepts of the regression line

Limit of Quantification

The LOQ is the lowest level that an analyte can be quantitated with any degree of certainty.

The LOQ can be determined by a signal-to-noise ratio of 10:1, or approximated by multiplying the LOD by 3.3. As with LOD, this function is easily obtained from current data-acquisition software. Similarly, LOQ can be estimated by the equation $LOQ = 10 (SD/S)$

Root MSE (SD) = the standard deviation of the y-intercepts of the regression

Specificity

Specificity is the ability of a method to discriminate between the analyte (s) of interest and other components that are present in the sample.

Placebo of the Clorsulon and Ivermectin Injection, equivalent to the sample weight was taken and solution prepared similarly to the sample solution. The solution was analyzed as per the proposed method. Sample solution was also analyzed as per the proposed method. No interference from placebo was observed at the retention time of the drugs peaks. The absence of a peak eluting at the retention time of the active ingredient is sufficient to demonstrate specificity for excipients.

Accuracy

Accuracy is the closeness in agreement between the accepted true value or a reference value and the measured result obtained. Accuracy studies are usually evaluated by determining the recovery of a spiked sample of the analyte into the matrix of the sample to be analyzed.

The accuracy of the assay method was evaluated with the recovery of the standards from excipients. Three different quantities (low, medium and high i.e. 80%, 100% and 120%) of the authentic standards were added to the placebo. Sample solutions are prepared in triplicate for each spike level as described in the test preparation.

Test Solution

An accurately weighed quantity of 20 mg of Ivermectin and 200 mg of Clorsulon were transferred into each of the three 100 ml volumetric flasks. 2 ml of Ivermectin plus (clorsulon 100 + Ivermectin 10 mg) placebo was added to the each. then methanol was added to the mark and the flasks were mixed and sonicated for 5 minutes to produce a solutions having a concentrations of 200 µg/ml of Ivermectin and 2000 µg/ml of Clorsulon.

An accurately 4, 5, and 6 milliliters of each test solutions

prepared in (1) in triplicate were Transferred to nine 50 ml volumetric flasks and made up to the volume with methanol to produce a solutions having a concentration levels of 80% (160 µg/ml of clorsulon, 16 µg/ml of ivermectin), 100% (200 µg/ml of clorsulon, 20 µg/ml of ivermectin) and 120% (240 µg/ml of clorsulon, 24 µg/ml of ivermectin) respectively.

Shake well and Filter through 0.45µl nylon filter and inject into the HPLC system.

Standard Solution

An accurately weighed quantity of 20 mg of Ivermectin and 200 mg of Clorsulon were transferred to a 100 ml volumetric flask. then methanol was to the mark and ultrasonicated for 5 minutes to produce a solution having a concentration of 200 µg/ml of Ivermectin and 2000 µg/ml of Clorsulon. 5 ml of this solution was diluted to 50 ml with methanol to produce a solution having the concentration of 20 µg/ml of Ivermectin and 200 µg/ml of Clorsulon.

Precision

Precision is a measure of the ability of the method to generate reproducible results. For the precision study, precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day) in triplicate. Repeatability refers to the use of the analytical procedure over a short period of time that was evaluated by assaying of six determinations at 100% of the test concentrations during the same day prepared in the manner described above in the Assay working Solution Page No (11). Intermediate precision was assessed by comparing the assay of six determinations at 100% of the test concentrations on different days (3 days) prepared in the same manner for repeatability.

Robustness

Robustness is a measure of the performance of a method when small, deliberate changes are made to the method conditions. The intent of this validation parameter is to identify which, if any, of the method conditions are the most critical to the successful performance of the method.

The Robustness was determined by injecting triplicate injections of standard and by assaying of six determinations at 100% of the test concentrations of the same Ivermectin plus Batch used in the precision Study.

Robustness of the method was checked by varying the instrumental conditions such as flow rate, Organic content in mobile phase ratio, wavelength of detection.

3. Results and Discussion

System Suitability

Table 1. Result of System suitability test.

Injection #	Ret. Time	Peak Area	Theo. Plate	Tailing Factor
1	1.726	1891931	1752.694	1.577
2	1.726	1892528	1770.867	1.599
3	1.726	1890773	1785.780	1.603
4	1.726	1890626	1814.087	1.609
5	1.726	1889911	1850.867	1.604
Average	1.726	1891153.8	1794.859	1.5984
STDEV	0	1056.0065	38.54395	0.012482
RSD	0	0.06	2.15	0.78

Table 2. Result of System suitability test.

Injection No.	Ret. Time	Peak Area	Theo. Plate	Tailing Factor	Resolution
1	6.612	394981	5309.001	0.977	18.510
2	6.598	394743	5390.561	0.978	18.612
3	6.586	394593	5493.684	0.978	18.735
4	6.572	394663	5621.079	0.979	18.900
5	6.557	393981	5724.026	0.980	19.051
Average	6.57825	394592.2	5557.338	0.97875	18.8245
STDEV	0.021517	168.8471	168.1153	0.00114	0.217541
RSD	0.33	0.04	3.03	0.12	1.15

Clorsulon Linearity

Table 3. Data for Calibration Curve for Clorsulon.

Percent Level	Concentration (X)	Peak Area Response (Y)
50%	100 µg/ml	918728.3
75%	150 µg/ml	136114
100%	200 µg/ml	1837846
125%	250 µg/ml	2313903
150%	300 µg/ml	2731636
175%	350 µg/ml	3236974
200%	400 µg/ml	3667866

X = Concentration of Clorsulon Standard in µg/ml.

y = Peak Area Response.

Clorsulon Concentration in µg/ml Vis Fitted Peak response Graph

Slope (b): 9197.802

y- Intercept (a): - 2869.502

Regression Equation(y): - 2869.502 + 9197.802

Figure 3 Calibration Curve of Clorsulon.**Table 4** Data summarize of regression of y and x parameters for Clorsulon.

No.	Parameter	Symbol	Value
1	R- Squared	r^2	0.9998
2	Slope coefficient (x)	A	-2869.502
3	Intercept (constant) coefficient	B	9197.802
4	Regression equation	$Y = a \cdot C + b$	$Y = -2869.502 + 9197.802$ $Y = 9197.802 - 2869.502 \cdot C$

Ivermectin Linearity

Table 5. Data for Calibration Curve for Ivermectin.

Percent Level	Concentration (X)	Peak Area Response (Y)
50%	10 µg/ml	191366.7
75%	15 µg/ml	286604
100%	20 µg/ml	385881
125%	25 µg/ml	487493.7
150%	30 µg/ml	576688
175%	35 µg/ml	684579.7
200%	40 µg/ml	777129.7

X = Concentration of Clorsulon Standard in µg/ml.

y = Peak Area Response.

Ivermectin Concentration in µg/ml Vis Fitted Peak response Graph

Slope (b): 19600.34

y - Intercept (a): - 5759.511

Regression Equation(y): - 5759.511 + 19600.34

Figure 4. Calibration Curve of Fitted Values of Ivermectin peak Areas.**Table 6.** Data summarize of regression of y and x parameters for Ivermectin.

No.	Parameter	Symbol	Value
1	R- Squared	r^2	0.9998
2	Slope coefficient (x)	A	19600.34
3	Intercept (constant) coefficient	B	-5759.511
4	Regression equation	$Y = a \cdot C + b$	$Y = 19600.34 - 5759.511$ $Y = -5759.511 + 19600.34 \cdot C$

Limit of Detection Calculations**Ivermectin Component**

The method based on the residual standard deviation of a regression line and slope.

The limit of detection and the limit of quantification of the drug were calculated using the following equations as per ICH guidelines.

$$LOD = 3.3(SD/S).$$

(SD)= the standard deviation of the response = Root MSE = 3620.8

$$(S)= \text{the slope of the calibration curve} = 19600.34$$

$$LOQ = 10(SD/S).$$

The standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

Root MSE is the standard deviation of the error term, and is the square root of the Mean Square Residual (or Error) =

$$SD = 3620.8$$

The limit of detection (LOD) and the limit of quantification (LOQ) of the drug were established by evaluating the minimum level at which the analyte could be readily detected and quantified.

$$LOD = 3.3 \times (3620.8/19600.34) = 0.61 \mu\text{g/ml}$$

$$LOQ = 10 \times (3620.8/19600.34) = 1.80 \mu\text{g/ml}$$

Clorsulon Component

The method based on the residual standard deviation of a regression line and slope.

The limit of detection and the limit of quantification of the drug were calculated using the following equations as per ICH guidelines.

$$LOD = 3.3(SD/S).$$

(SD)= the standard deviation of the response = Root MSE = 17180

$$(S)= \text{the slope of the calibration curve} = 9197.802$$

$LOQ = 10 (SD/S)$.

The standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

Root MSE is the standard deviation of the error term, and is the square root of the Mean Square Residual (or Error) = $SD = 17180$

The limit of detection (LOD) and the limit of quantification (LOQ) of the drug were established by evaluating the minimum level at which the analyte could be readily detected and quantified.

$$LOD = 3.3 \times (17180 / 9197.802) = 6.16 \mu\text{g/ml}$$

$$LOQ = 10 \times (17180 / 9197.802) = 18.68 \mu\text{g/ml}$$

Specificity

Figure 5. Above shows Placebo chromatogram.

Figure 6. Above shows the combined drug Sample (Ivermectin + Clorsulon) chromatogram.

Chromatogram of Placebo and Sample for Specificity Figure 5 shows a Placebo chromatogram demonstrating the absence of a peak for the main component for an injection of a mixture of the excipients.

Accuracy

Table 7. Results of recovery of Clorsulon from spiked placebo.

Spike level (%)	Average 'µg/ml' added	Average 'µg/ml' found	Mean% recovery	RSD (%)
80%	160 µg/ml	159.98 µg /ml	99.98	1.19
100%	200 µg/ml	199.7 µg /ml	99.85	0.65
120%	240 µg/ml	239.83 µg /ml	99.93	0.59
Average Recovery%= 99.92%				
Mean RSD%= 0.06%				

*Acceptance Criteria: Recovery% is 98 – 102%.

Table 8. Results of recovery of Ivermectin from spiked placebo.

Spike level (%)	Average 'µg/ml' added	Average 'µg/ml' found	Mean% recovery	RSD (%)
80%	16 µg/ml	15.86 µg /ml	99.14	0.94
100%	20 µg/ml	19.9 µg /ml	99.52	0.43
120%	24 µg/ml	23.91 µg /ml	99.64	0.70
Average Recovery%=99.43%				
Mean RSD%=0.26%				

*Acceptance Criteria: Recovery% is 98 – 102%.

Table 9. Results of the intra-day precision test (repeatability).

No.	Clorsulon Assay Results
Assay-1	98.57%
Assay-2	97.75%
Assay-3	97.49%
Assay-4	98.10%
Assay-5	97.13%
Assay-6	97.54%
Assay-7	97.28%
Assay-8	97.23%
Average	97.50%
STDEV	0.33
RSD%	0.34

*Acceptance Criteria: RSD ≤ 2.

Table 10. Results of the intra-day precision test (repeatability).

No.	Ivermectin Assay Results
Assay-1	98.64%
Assay-2	97.62%
Assay-3	97.34%
Assay-4	97.94%
Assay-5	96.89%
Assay-6	97.44%
Assay-7	97.08%
Assay-8	96.96%
Average	97.48%
STDEV	0.58
RSD%	0.59

*Acceptance Criteria: RSD ≤ 2.

Intermediate Precision

Table 11. Results of the inter-day precision test (intermediate precision).

Replicates	Clorsulon Assay Results		
	Day-1	Day-2	Day-3
Assay-1	100.22%	98.63%	99.93%
Assay-2	100.22%	98.18%	97.98%
Assay-3	100.94%	99.02%	98.07%
Assay-4	98.66%	98.56%	98.37%
Assay-5	98.02%	99.02%	98.09%
Assay-6	100.16%	98.96%	97.35%

Replicates	Clorsulon Assay Results		
	Day-1	Day-2	Day-3
Average	99.7%	98.7%	98.5%
STDEV	1.11	0.33	0.86
RSD%	1.11	0.33	0.88

Table 12. Results of Overall inter-day precision test (intermediate precision).

Assay	% Content
Day-1	99.7
Day-2	98.7
Day-3	98.5
Average	98.9
STDEV	0.64
RSD%	0.65

*Acceptance Criteria: RSD ≤ 2.

Intermediate Precision

Table 13. Results of the inter-day precision test (intermediate precision).

Replicates	Ivermectin Assay Results		
	Day-1	Day-2	Day-3
Assay-1	97.8%	99.3%	99.0%
Assay-2	97.5%	99.3%	95.9%
Assay-3	97.9%	100%	96.6%
Assay-4	97.3%	98.1%	97.3%
Assay-5	98.3%	97.1%	96.6%
Assay-6	97.5%	99.6%	95.7%
Average	97.7%	98.9%	96.9%
STDEV	0.36	1.08	1.19
RSD%	0.37	1.09	1.23

Table 14. Results of Overall inter-day precision test (intermediate precision).

Assay	% Content
Day-1	97.7
Day-2	98.9
Day-3	96.9
Average	97.8
STDEV	1.0
RSD%	1.0

*Acceptance Criteria: RSD ≤ 2.

Robustness

Table 15. Summary of Robustness Parameters.

Parameter Changed	Clorsulon	Ivermectin	Present method	
			Clorsulon	Ivermectin
Wavelength 246 nm	98.6	98.3	98.5	98.6
	97.8	97.3	97.7	97.6
	97.5	97.0	97.4	97.3
	98.2	97.6	98.1	97.9
	97.4	97.1	97.5	97.4
	97.6	96.8	97.2	97.0
Average	97.9	97.4	97.7	97.6
STDEV	0.46	0.54	0.48	0.56
RSD%	0.47	0.55	0.49	0.57

(Overall RSD) *** Acceptance Criteria: RSD $\leq$ 2%.**Table 16.** Summary of Robustness Parameters.

Parameter changed	Clorsulon	Ivermectin	Present Method as Standard	
			Clorsulon	Ivermectin
Flow rate 0.8 ml/ min	97.6	97.1	98.5	98.6
	97.4	96.7	97.7	97.6
	97.9	97.3	97.4	97.3
	97.2	96.9	98.1	97.9
	97.3	96.8	97.5	97.4
	97.1	96.5	97.2	97.0
Average	97.4	96.9	97.7	97.6
STDEV	0.29	0.28	0.48	0.56
RSD%	0.30	0.29	0.49	0.57

(Overall RSD) *** Acceptance Criteria: RSD $\leq$ 2%.**Table 17.** Summary of Robustness Parameters.

Parameter changed	Clorsulon	Ivermectin	Present method	
			Clorsulon	Ivermectin
Mobile phase ratio (50:40:10: v/v/v) Aceto: MeOH: PW	99.9	99.3	98.5	98.6
	98.9	97.7	97.7	97.6
	98.4	97.7	97.4	97.3
	98.2	97.8	98.1	97.9
	98.4	97.3	97.5	97.4
	98.2	96.8	97.2	97.0
Average	98.7	97.8	97.7	97.6
STDEV	0.65	0.84	0.48	0.56
RSD%	0.66	0.86	0.49	0.57

(Overall RSD) *** Acceptance Criteria: RSD $\leq$ 2%.**Table 18.** Summary of Robustness Parameters.

Parameters	RT $\pm$ SD	Theo. plates $\pm$ SD	Tailing Factor
Present Method	1.72 $\pm$ 0.1	1520.49 $\pm$ 1.2	1.43
246 nm	1.72 $\pm$ 0.05	1539.78 $\pm$ 1.4	1.54
0.8 ml /min	2.14 $\pm$ 0.12	1701.04 $\pm$ 7.2	1.44
Changed M. phase ratio	1.72 $\pm$ 0.2	2191.6 $\pm$ 2.9	1.530

Table 19. Summary of Robustness Parameters Ivermectin.

Parameters	RT $\pm$ SD	Resolution $\pm$ SD	Theo. plates $\pm$ SD	Tailing Factor
Present Method	6.91 $\pm$ 0.2	20.55 $\pm$ 0.3	7106.94 $\pm$ 0.1	1.06
246 nm	6.96 $\pm$ 0.4	20.26 $\pm$ 3.09	6742.26 $\pm$ 6.05	1.03
0.8 ml /min	9.02 $\pm$ 0.2	22.49 $\pm$ 1.1	8014.90 $\pm$ 0.6	1.05
Changed M. phase ratio	6.45 $\pm$ 0.7	20.25 $\pm$ 0.8	6528.74 $\pm$ 1.1	1.03

Force degradation Study

Table 20. Summary result of the Force degradation.

Parameters	Assay%	
	CLO	IVM
Present Method No Stress (as STD)	97.50	97.48
Thermal degradation	97.71	96.58
Acid degradation	94.72	78.59
Base degradation	94.23	72.88
Oxidation degradation	97.57	91.70
Photo degradation (UV)	96.76	96.19

Discussion

In this work an analytical HPLC method for simultaneous determination of Ivermectin plus injection (Ivermectin + Clorsulon) was developed and validated. The basic chromatographic conditions were designed to be simple and easy to use and reproduce and were selected after testing the different conditions that affect HPLC analysis, for example column, aqueous and organic components of the mobile phase, proportion of mobile phase components, detection wavelength, diluents and concentration of analyte.

To obtain better separation with good resolution these Chromatographic conditions were optimized using a stainless steel column (Thermo BDS C 18, 150mm x 4.6mm 5.0 μ m), which was maintained at Ambient Temperature. The analytical wavelength was set at 245 nm and samples of 10. μ l were injected to HPLC system. The mobile phase consisting of a mixture of Acetonitrile: Methanol: Purified Water in the ratio (60:30:10), at a flow rate of 1ml/min. The mobile phase was filtered through 0.45 μ m filter and degassed for 10 minutes by sonication.

To determine linearity a calibration graph was obtained by plotting Ivermectin + Clorsulon injection concentration against peak area. The method was linear in the range of (10 – 40 μ g/ml) and (100 – 400 μ g/ml) for Ivermectin and Clorsulon concentrations respectively with a correlation coefficient 0.9998 for Ivermectin and 0.9998 for Clorsulon for more detail refer to tables 3 to 6 and figures 3&4.

To determine the Specificity which is the ability of a method to discriminate between the analyte (s) of interest and other components that are present in the sample. The method shows no interference from placebo was observed at the retention time of the drugs peaks. (See Figure 5 and 6.

The accuracy of the method was assessed by determination of recovery for three concentrations covering the range of the method. The amount of Ivermectin and Clorsulon were recovered in the presence of placebo interference, were calculated. The mean recovery of Ivermectin and Clorsulon were 99.43% and 99.92% respectively which is satisfactory and result was shown in Table 7 and 8.

Precision of the method was done; the% RSD for repeatability (Intra-day precision) were 0.34% and 0.59% for Ivermectin and Clorsulon respectively. The% RSD for intermediate precision were 0.65% and 1.0% for Clorsulon and Ivermectin respectively, Tables 11 to 14 explain the results of the inter-day precision test (intermediate precision). The method was found to be precise Since the RSD% is less

than 2.0%.

The robustness of the method was assessed by assaying test solutions under different analytical conditions deliberately changed from the original conditions such as wavelength, flow rate and mobile phase ratio. Assay and system suitability parameters of changed method conditions compared with the original method proves that the analytical method remained unaffected by slight but deliberate changes in the analytical conditions therefore the method is robust. See the results in tables 15, 16, 17, 18 and 19.

A stability-indicating ability of the method is studied by a deliberate degradation through exposure of Ivermectin plus (Clorsulon 100 + Ivermectin 10 mg) to acid hydrolysis, base hydrolysis, photo degradation (U V), heat and Oxidation. The acid and base hydrolysis are significantly affect the two drugs and the resulting degradants are very remarkable in the chromatograms while the other degrading substances show no or very slight influence on the two drugs for details refer to Table 20.

This furnished evidence that the method is suitable for its intended purpose.

4. Conclusion

The intensive approach described in this manuscript was used to develop and validate a liquid chromatographic analytical method that can be used for simultaneous determination of Ivermectin and Clorsulon in a pharmaceutical dosage form (injection). This HPLC -method for simultaneous estimation of the combined drug was successfully developed and validated for its intended purpose. The method clearly proves to be specific, linear, precise, accurate, robust and Stability-Indicating.

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