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Development and Validation of HPTLC and HPLC Methods for Simultaneous Determination of Closantel and Ivermectin in Veterinary Drug Products

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ABSTRACT

Two validated chromatographic methods for simultaneous determination of Closantel (CLS) and Ivermectin (IVR) in veterinary drug products have been proposed. The first method was a simple HPTLC method where separation was performed on HPTLC silica gel 60 F_{254} plates using toluene: isopropanol: ammonia 33%: glacial acetic acid (70:28:10:1, by volume) as a developing system, Rf values were found to be 0.35 and 0.65 for CLS and IVR, respectively. The second method was an isocratic HPLC method where separation was performed on a C18 column using acetonitrile: methanol: 5mM ammonium dihydrogen phosphate buffer PH 6 (60:30:10, by volume) as a mobile phase. Retention times were found to be 1.2 min and 2.6 min, respectively. The linear ranges of the first method were found to be 0.2-12 µg band^{-1} and 0.06-3 µg band^{-1}; those of the second method were found to be 0.5 -500 µg mL^{-1} and 0.5-200 µg mL^{-1} for CLS and IVR, respectively. Both methods were validated according to the ICH guidelines and applied for the determination of the two drugs in drug substance and drug products without interference from reported excipients.

Keywords: Closantel; Ivermectin; HPTLC; Densitometry; HPLC

INTRODUCTION

Closantel (CLS) (figure 1a) chemically is a [5-Chloro-4-(4-chlorophenyl) cyanomethyl]-2-methylphenyl]-2-hydroxy-3, 5-diiodobenzamide. It is a salicylanilide family member and one of the most extensively used fasciolicidal drugs [1]. Literature review for CLS determination in drug substances revealed a few chromatographic and spectroscopic methods [2-6].

Ivermectin (IVR) (figure 1b) is a mixture of not less than 80% 22, 23dihydro avermectin B1a and not more than 20% 22, 23 dihydro avermectin B1b. It is effective against a wide range of helminthes [7]. Literature review revealed that a few analysts determined it singly in pharmaceutical formulations or in mixtures with other drugs [8-11].

The combined drug product is used for the treatment of mixed trematode (fluke) and nematode or arthropod infestations due to gastrointestinal roundworms, lungworms, eyeworms, warbles, mites and lice of cattle and sheep [12].

Literature review for simultaneous determination of CLS and IVR in drug products revealed that only one HPLC determination method has been reported [13].

The objective of this work was to develop a simple HPTLC simultaneous determination method for CLS and IVR as to the best of our knowledge no HPTLC method has been reported to fulfil that target, in addition to a rapid simple yet, accurate isocratic HPLC simultaneous determination method for both CLS and IVR with no need for prior separation or interference from reported excipients.

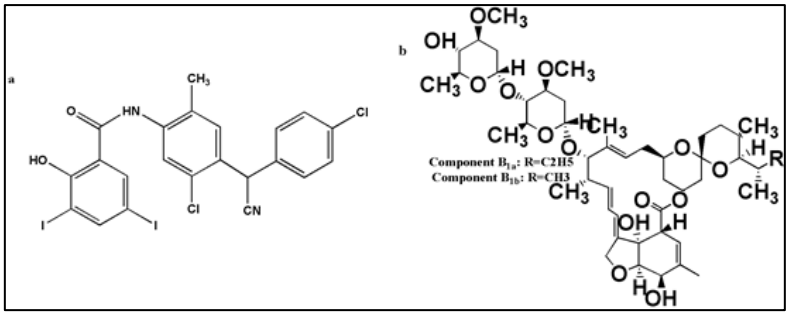

Figure 1: Chemical structures of (a) Closantel (b) Ivermectin

EXPERIMENTAL SECTION

Instruments

An HPTLC system consists of a CAMAG® TLC Densitometer (SN: 130407) connected to connected to a Fujitsu® desktop computer with WinCATS software (Version 1.2.0) and CAMAG Linomat IV auto sampler (Muttenz, Switzerland) with a CAMAG® micro syringe (25µL), ADC2 chromatographic chamber and HPTLC plates [20 cm x 10 cm, 0.20 mm] coated with silica gel 60 F_{254} [EMD Millipore, supplied by Sigma Aldrich].
An HPLC system consists of an Agilent® 1260 HPLC/UV instrument connected to an HP desktop computer and controlled by Agilent chemstation software for HPLC equipped with a quaternary pump, Rheodyne® injector with a 20 µL loop and a UV variable wavelength detector (Minnesota, USA) . Separation was done on a Spherisorb ODS2 RP column (150 × 4.6 mm, 5 µm particle size). The samples were injected by the aid of a 100 µL Hamilton® analytical syringe.

Materials and reagents

Pure standard:

CLS was a certified standard kindly supplied by Norbrook agent in Egypt (Egavet). Its purity was certified to be 99.40. IVR standard was a USP certified reference standard and was supplied by Sigma Aldrich, Egypt. It was certified to be 90 %.

Pharmaceutical formulation:

Closamectin® vials, label claim: 125 mg CLS and 5 mg IVR per 1 mL manufactured by Norbrook UK was kindly supplied by Egavet.

Chemicals and reagents:

Methanol and acetonitrile (HPLC grade), isopropanol and ethyl acetate (analytical grade) were obtained from Sigma Aldrich, Cairo, Egypt. Ammonia solution 33%, toluene, ammonium dihydrogen orthophosphate and glacial acetic acid were obtained from Adwic, Cairo, Egypt.

Procedure

Preparation of stock solutions:

Stock standard solutions A and B of 10 mg mL^{-1} and 1 mg mL^{-1} for CLS and IVR respectively were prepared in acetonitrile for the HPTLC method.
Stock standard solutions C and D of 5 mg mL^{-1} and 1 mg mL^{-1} for CLS and IVR respectively were prepared in mobile phase for HPLC method.

HPTLC method

Construction of calibration curve:

Aliquots of stock standard solutions A and B were transferred into a series of 10 mL volumetric flasks to give concentration ranges of 1-45 µg mL^{-1} and 0.4-1.8µg mL^{-1} for CLS and IVR, respectively. A volume of 5µL of each solution was applied in triplicates to the HPTLC plates as 5mm bands using Linomat IV applicator to give ranges equivalent to 0.2-12µg $band^{-1}$ and 0.06-3µg $band^{-1}$ for CLS and IVR respectively by a dosage speed of 75 nL S^{-1}, the bands were applied 10 mm apart and 15 mm from the bottom edge of the plate. Linear ascending development was performed in the ADC2 chromatographic chamber using a development system consisting of toluene: isopropanol: ammonia: glacial acetic acid(70: 28: 10: 1, by volume) previously saturated with 25mL of the same system for 25 min. at room temperature and 40% relative humidity. The developed plates were dried in an air stream and scanned at 245 nm using Camag® scanner 3 densitometer employing the deuterium lamp, absorbance mode at 6 mm x 0.6 mm slit dimension and scanning speed of 20 mmS^{-1}. Calibration curves relating

the optical density of each band to the corresponding concentration of CLS and IVR were constructed. The regression equations were then computed and used for determination of unknown samples.

HPLC method

Construction of calibration curve:

Aliquots from stock standard solutions C and D separately were transferred into a series of 10mL volumetric flasks. The contents of each flask were completed with the mobile phase to get concentration ranges of 0.5–500 and 0.5–200 µg mL^{-1} for CLS and IVR respectively .The samples were then chromatographed on reversed phase Spherisorb ODS2 C 18 RP column (150 × 4.6 mm, 5 µm particle size using acetonitrile: methanol: 5mMammonium dihydrogen phosphate buffer pH 6 (60:30:10, by volume) as a mobile phase. The mobile phase was filtered through Millipore filter 0.45 µm, white nylon HNWP 47 mm and was degassed for 15 min in an ultrasonic bath prior to use. UV detection was done at 245 nm. The system was operated at 25 °C. The flow rate was isocratic at 1mL/min. The samples were filtered through a 0.45 µm membrane filter, and 20 µL were injected by the aid of an Agilent analytical syringe. The chromatograms were recorded, the peak areas of each drug were determined and the calibration curves relating peak areas to the corresponding concentrations for IVR and CLS were constructed and used for determining concentration of unknown samples.

Preparation of laboratory prepared mixture solutions:

Laboratory prepared mixture solutions containing different ratios of CLS and IVR were prepared by diluting and mixing different aliquots from CLS and IVR stock solutions into a series of 10 mL volumetric flasks, and steps were proceeded as mentioned under each method, the concentrations were then calculated from the corresponding regression equations.

Preparation of sample solutions:

Stock sample solution was prepared by mixing three vials of Closamectin® and transferring a10 mL aliquot to a 100 mL volumetric flask and dissolved in 50 mL acetonitrile by the aid of an ultrasonic bath for 5 minutes and the volume was completed to the mark with the same solvent. Working sample solutions were prepared by appropriate dilution of stock sample solution and steps were proceeded as mentioned under each method.

RESULTS AND DISCUSSION

This manuscript describes for the first time a simple HPTLC simultaneous determination method in addition to a rapid isocratic HPLC method suitable for routine quality inspection of CLS and IVR in drug substances or drug products with no need for prior separation or interference from reported excipients.

HPTLC method optimization

Studying physicochemical properties of CLS and IVR was the first guideline for starting the selection of developing system components. So first, a mixture of toluene: ethyl acetate was tried (97:03 v/v) but the results were not satisfactory as IVR eluted at Rf 0.3 while CLS remained at baseline. Then, polarity was increase by trying toluene: methanol (75:25 v/v) but both drugs were coeluted at solventfront. So, a medium polarity system was tried consisted of toluene: isopropanol (70:30 v/v), this resulted in eluting of CLS and IVR at 0.55 and 0.57 respectively with a very bad resolution. So changing PH was tried, so a mixture of toluene: isopropanol: aqueous ammonia 33% (70:29: 10) but CLS and IVR eluted at 0.4 and 0.43 with bad resolution. Finally, the system toluene: isopropanol: ammonia 33%: glacial acetic acid (70: 28: 10: 1) resulted in good resolution as CLS and IVR eluted at Rf 0.35 and 0.65 respectively (Figure 2). The maximum absorption wavelength for IVR was selected to be the measurement wavelength (245 nm)as being the minor component in the drug product it was important to get the most benefit of the measurement conditions to obtain the highest sensitivity for it.

HPLC method optimization

Studying physicochemical properties of CLS and IVR was also the first guideline for starting to select the components of mobile phase and the type of chromatographic column. The pka values for CLS and IVR are reported to be 4.2 and 6.5 respectively. So a Spherisorb ODS2 column was selected as a general purpose, silica based, reversed phase C18 column, the ODS 2 packing features intermediate ligand density. For mobile phase, first; a simple mixture of acetonitrile: water (90:10) was tried and resulted in a poor separation for CLS and IVR, then; the USP [5] mobile phase used for determination of IVR was tried which consists of acetonitrile: methanol: water (53:27.5:19.5) and resulted in a forked asymmetric peak for CLS so it was found that the pH adjustment is an important factor so, the use of acetonitrile: methanol: 5mM ammonium dihydrogen phosphate buffer pH 6 (60:30:10, v/v) as a mobile phase resulted in satisfactory separation and peak symmetry for CLS and IVR at 1.2 min and 2.6 min, respectively (Figure 3) . Best separation was obtained on a flow rate 1mL/min. this method is advantageous to the reported HPLC method as more sensitive and faster elution is achieved.

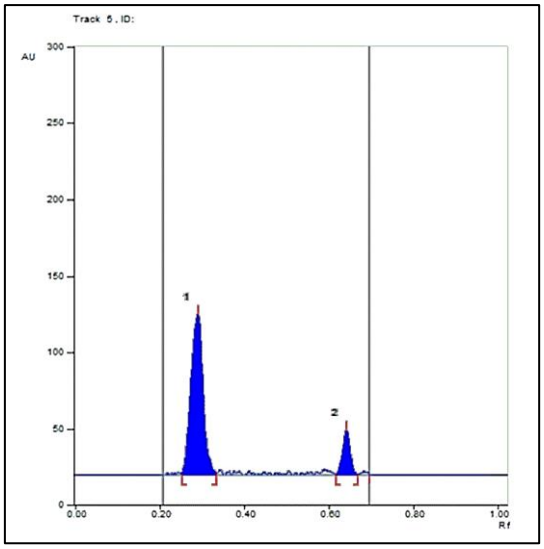

Figure 2: HPTLC Densitogram of CLS (1) at Rf 0.35 and IVR (2) at Rf 0.65

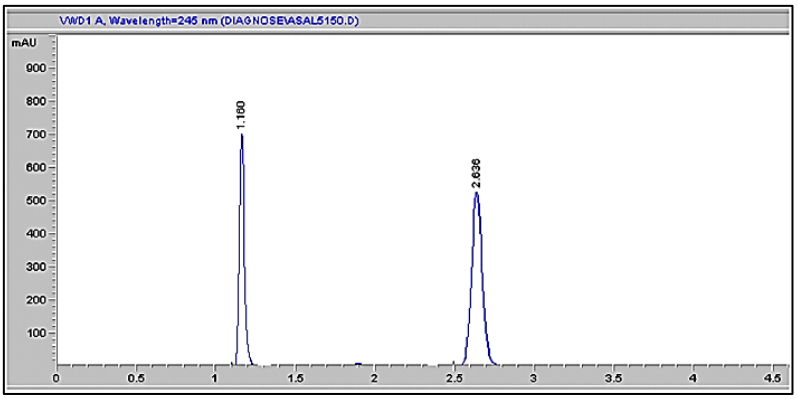

Figure 3: HPLC chromatogram of CLS and IVR at Rt 1.2 min. and 2.6 min. respectively

Methods validation

Specificity:

It was ascertained by analyzing different laboratory mixtures containing CLS and IVR in the presence of pharmaceutical excipients and comparing retention factor and area to those of certified standard solutions. Satisfactory results were obtained indicating the high selectivity of the proposed methods. Recovery of CLS and IVR in laboratory prepared mixtures containing dosage form excipients was calculated to express specificity (Table 1).

Linearity and range

For HPTLC method: Under the specified experimental conditions, the relationships between concentrations of selected drugs and peak areas of the bands were investigated and found to be linear in the range of 0.2-12µg band^{-1} and 0.06-3µg band^{-1} for CLS and IVR respectively. The regression equations were computed and found to be:

$$P_{CLS}=3598C+6.80 \quad r=1$$

$$P_{IVR}=3953C+2.81 \quad r=0.9995$$

Where P is the peak area, C is the concentration in µg band^{-1}**;** r is the correlation coefficient (Table 1).

For HPLC method: Under the specified experimental conditions, the relationships between concentrations of selected drugs and peak areas were investigated and found to be linear in the range of 0.5-500µg mL^{-1} and 0.5-200µg mL^{-1} for CLS and IVR respectively. The regression equations were computed and found to be:

$$P_{CLS}=41.43C+0.333 \quad r=1$$

$$P_{IVR}=29.37C+0.009 \quad r=1$$

Where P is the peak area, C is the concentration in µg mL^{-1}**;** r is the correlation coefficient (Table 1).

Precision:

The precision of the proposed methods was assessed by performing intraday and interday variation studies. In the intraday studies, standard and sample solutions were analyzed in triplicate on the same day and % RSD was calculated. In case of interday studies, standard and sample solutions were analyzed in triplicate on three consecutive days and % RSD were calculated (Table 1).

Table 1: Analytical parameters and validation results of the determination of CLS and IVR by the proposed methods

	HPTLC method		HPLC method	
Method parameter	**Closantel**	**Ivermectin**	**Closantel**	**Ivermectin**
Wavelength(nm)	245	245	245	245
Linearity range	0.2-12µg/band	0.06-3µg/band	0.5-500 µg/mL	0.5-200µg/mL
Time of analysis (min/run)	30		5	
Linearity				
Intercept	6.8	2.81	0.333	0.009
Slope	3598	3953	1	29.37
Correlation coefficient(r)	1	0.9995	41.43	1
Accuracy(mean ±%RSD)				
Low conc.	99.75±0.3	98.70±0.5	100.1±0.15	99.3±0.15
Medium conc.	98.70±0.3	100.03±0.3	98.65±0.10	100.2±0.26
High conc.	99.05±0.5	100.09±0.2	99.8±0.27	99.8±0.25
Specificity [a]	101.5±1.2	100.1±1.0	100.35±0.40	100.4±0.65
Precision				
(±%RSD)[b]	±0.62	±0.32	±0.15	± 0.08
(±%RSD)[c]	±0.95	±0.78	±0.68	±0.45
Robustness	±0.58	±0.21	±0.05	±0.03
LOD[d]	0.022 µg/band	0.013 µg/band	0.12µg/mL	0.058 µg/mL
LOQ[d]	0.066 µg/band	0.039 µg/band	0.36 µg/mL	0.178 µg/mL

a Recovery of CLS and IVR in laboratory prepared mixtures containing dosage form excipients; b Intraday precision (average of 3 different concentrations of / 3 replicate each (n = 9) within the same day); c Interday precision (average of 3 different concentrations of / 3 replicate each (n = 9) repeated on 3 successive days); d Calculated from equation [LOD = 3.3 (S.D / S), LOQ = 10 (S. D / S); where S.D is the residual standard deviation of the slope and S is the slope for HPTLC and HPLC methods.

Robustness:

For HPTLC: It was checked by investigating the effect of small deliberate changes in the experimental conditions on separated spots. Mixtures of CLS and IVR were separated under different conditions by using different volumes of developing system by ±10%, different saturation times by ±20% and different toluene composition by ± 5% in the developing system. The Rf values of the separated bands using the mentioned volumes of developing system range did not change, while changing toluene composition and saturation times was accompanied by slight decrease or increase of Rf of the two peaks. This did not affect separation (Table 1).

For HPLC method: Mixtures of CLS and IVR were separated under different conditions by using different pH values 6.0 ± 0.2, different flow rates (1.0 ± 0.2 mL/min) and different acetonitrile composition by 60 ± 5% of the mobile phase. The Rt values of the separated peaks using the mentioned pH range did not change, while changing the flow rate and mobile phase was accompanied by slight decrease or increase of Rt of the two peaks. However, the calculated resolution (R) values were always above 2, ensuring complete separation .Other parameters such as capacity factor were shown in Tables 2 and 3.

Table 2: System suitability testing Parameters of HPTLC method

Parameter	CLS	IVR
K' (capacity factor)	5.5	2.5
α(Relative retention)	3	
Resolution	3.33	
Symmetry factor	0.99	1.09

Table 3: System suitability testing parameters of HPLC method

Parameter	CLS	IVR
Resolution	6.11	
α(Relative retention)	6.2	
K' (capacity factor)	5	31
N (column efficiency)	5773	2755
HETP	2.59×10^{-3}	5.44×10^{-3}
T (Tailing factor)	1	1.09

Recovery:

The suggested methods were successfully applied for determination of CLS and IVR in their drug product (Closamectin® vial). The results were satisfactory and with good agreement with the labeled amounts. Applying

the standard addition technique, no interference due to excipients was observed as shown from the results in Tables 4 and 5.

Table 4: Analysis of CLS and IVR in marketed formulations by HPTLC method and application of standard addition technique

Standard addition						
Product	**proposed method %recovery**	**taken amount**	**added amount**	**total found***	**standard found***	**%recovery of added amount**
Closamectin® vial (each 1mL contains 125mg CLS and 5mg IVR)	**CLS** 100.57± 0.99 (Mean ±RSD *)	2.5	0	2.5±0.01	-	-
		2.5	1	3.51±0.01	1.01±0.01	101.00± 0.99
		2.5	2	4.51±0.02	2.01±0.02	100.5± 0.99
		2.5	5	7.51±0.05	5.01±0.05	100.2.±0.99
	IVR 100.23±0.99 (Mean ±RSD *)	0.5	0	0.523±0.005	-	-
		0.5	0.25	0. 774±0.003	0.251±0.003	100.4±1.19
		0.5	0.5	1.02±0.005	0.497±0.005	99.4±1.01
		0.5	0.75	1.28±0.006	0.757±0.006	100.9±0.79

*Average of three determinations

Table 5: Analysis of CLS and IVR in marketed formulations by HPLC method and application of standard addition technique

Standard addition						
Product	**Proposed method %recovery**	**taken amount**	**added amount**	**total found***	**standard found***	**%recovery of added amount**
Closamectin® vial (each 1mL contains 125mg CLS and 5mg IVR)	**CLS** 100.3±0.5 (Mean ±RSD *)	125	0	125±0.02	-	-
		125	62.5	187.5±0.03	62.5±0.03	100.0±0.05
		125	187.5	312±0.1	187.5±0.1	100.0±0.53
		125	125	250±0.2	125±0.2	100.0±0.16
	IVR 100.4±0.1 (Mean ±RSD *)	5	0	5±0.02	-	-
		5	2.5	7.5±0.01	2.5±0.01	100.0±0.04
		5	5	10±0.01	5.0±0.01	100.0±0.02
		5	7.5	12.5±0.02	7.5±0.02	100.0±0.27

*Average of three determinations

LOD and LOQ

LOD and LOQ are assessed to determine the sensitivity of the method; their values are indicated in Table 1.

CONCLUSION

The proposed chromatographic methods are found to be accurate, rapid and reproducible so can be used efficiently for routine quality inspection of Closantel and Ivermectin in bulk, single or combined dosage forms with no interference from excipients.

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