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HPLC AND HPTLC SIMULTANEOUS DETERMINATION METHODS OF TRICLABENDAZOLE AND MOXIDECTIN IN COMBINED DOSAGE FORMS

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ABSTRACT

This manuscript describes two simple validated chromatographic methods for simultaneous determination of Triclabendazole (TCL) and Moxidectin (MOX) in combined dosage forms with no prior separation or interference from excipients. The first method was an isocratic HPLC method on a BDS phenyl C18 column using acetonitrile: methanol: 5mM ammonium dihydrogen phosphate solution (60:30:10, by volume) as a mobile phase at wavelength 242nm, retention times were found to be 1.9 min and 3.9 min for TCL and MOX, respectively. The second method was a simple HPTLC method where separation was performed on HPTLC silica gel 60 F_{254} plates using ethyl acetate: toluene: formic acid 85%: (50:45: 5, by volume) as a developing system, the developed bands were scanned at 242nm, Rf values were found to be 0.60 and 0.90 for TCL and MOX, respectively. The linear ranges of the first method were found to be 1-200 µg/mL and 0.5-100 µg/mL, while those of the second method were found to be 0.5 -20 µg/band and 0.1-2 µg/band for TCL and MOX, respectively. Both methods were validated and applied for the determination of the two drugs in pure raw material and combined dosage form with no interference from reported excipients and were found to be suitable for quality inspection of combined dosage forms.

KEYWORDS: HPLC, HPTLC, Triclabendazole, Moxidectin.

INTRODUCTION

Triclabendazole (**TCL**) is a 5-Chloro-6-(2, 3-dichlorophenoxy)-2-(methyl thio) benzimidazole. Triclabendazole is a benzimidazole anthelmintic used in veterinary medicine

for the treatment of fascioliasis and is investigation for the treatment of human paragonimiasis.[1]

Moxidectin (**MOX**) is a (6R, 15S)-5-O-Demethyl-28-deoxy-25-[(E)-1, 3 dimethylbut-1-enyl]-628-epoxy-23-oxomilbemycin B (E)-(23 Omethyloxime. It is an anthelmintic used in veterinary medicine. It is also used as a systemic veterinary ectoparasiticide and is under investigation for the treatment of human onchocrciasis.[2] Chemical structures of TCL and MOX are represented in Figure 1.

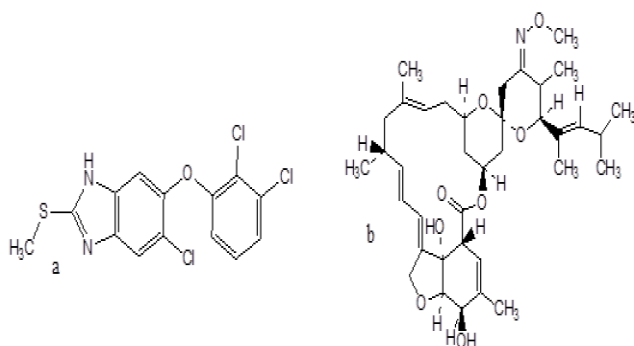

Figure 1: Chemical structure of TCL (a) and MOX (b)

The combination is used for the treatment and control of the gastrointestinal roundworms, lungworm and liver flukes in sheep and cattle. The product also showed effectiveness against certain benzimidazole resistant strains.[3]

Literature review for determination of TCL in bulk or dosage forms revealed some spectroscopic methods and an HPLC chromatographic method.[4-8]

While surveying the literature of MOX for the same purpose revealed only a recent spectroscopic method.[9]

To the best of our knowledge no analytical methods have been reported for simultaneous determination of the two drugs as a mixture in their combined pharmaceutical dosage forms. This may be due to the presence of TCL and MOX in a challenging ratio (50:1) in addition to a list of pharmaceutical excipients in the commercially available combined drug product. So, the main objective of this work was to develop selective, rapid, simple and precise validated chromatographic methods for simultaneous determination of both drugs in this ratio with no interference from excipients.

MATERIALS AND METHODS

Chemicals and reagents

Methanol, acetonitrile and ethyl acetate (HPLC grade), were obtained from Sigma Aldrich, Cairo, Egypt. Toluene, ammonium dihydrogen orthophosphate and formic acid 85% (analytical grade) were obtained from Adwic, Cairo, Egypt. The HPLC grade water was freshly obtained from Aquatron® double distillation and filtered through a 0.45 µm membrane filter .TCL and MOX working standards were kindly supplied by Zoetis office, Egypt. Their purities were certified to be 99.8% and 95.6%, respectively. Cydectin Triclamox sheep oral drench®, label claim: 50 mg TCL and 1 mg MOX per 1 mL manufactured by Zoetis UK Limited were obtained from Zoetis office, Egypt.

Instrumentation

For HPLC

An Agilent® 1260 liquid chromatographic system with a quaternary pump system (SN: DEAB 80855), an ultra violet variable wavelength (SN: DEABB 05675) detector and a Rheodyne injector (SN: DEABG 02069) equipped with 20 µL injector loop Agilent (Minnesota, USA) and connected to an HP® desktop computer. All solutions used for HPLC were filtered through 0.45µm Sartorius membrane filters and degassed using ultrasonic vibrations J.P Selecta (Barcelona, Spain) separation was done on a Hypersil BDS phenyl C18 column (250 × 4.6 mm, 5 µm particle size).

For HPTLC

HPTLC system (CAMAG®, Muttenz, Switzerland) which consists of a CAMAG 25 µL syringe, sample applicator (Linomat VI), CAMAG Automatic Developing Chamber 2(ADC2) and a densitometric CAMAG TLC scanner III (SN: 130407) operated by Win CATS software (Version 1.2.0) connected to a Fujitsu® desktop computer and the used plates are HPTLC aluminum sheets silica gel 60 F254 (20×10 cm, 0.2 mm), Merck Millipore.

Glassware

All the glassware used in the study was of class A Pyrex®.

Chromatographic Conditions

HPLC method

The mobile phase was prepared by mixing acetonitrile: methanol: 5mM ammonium dihydrogen phosphate buffer pH 6 (60:30:10, by volume). 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. All determinations were performed in an air conditioned laboratory atmosphere (18±2 ℃) using the following chromatographic conditions: Hypersil BDS phenyl C18 column (250 × 4.6 mm, 5 µm particle size), flow rate: 1mL/min, injection volume: 20µL, wavelength: 242nm.

HPTLC method

For detection and determination, aliquots were applied as separate bands 10mm apart and 15mm from the bottom of the plates, 5mm band length and 70nL/S dosage speed. The chromatographic tank was saturated with the developing system for 30 minutes prior to use. The plates were developed over a distance of 95mm in an ascending manner, air dried, and scanned under the following conditions: Deuterium lamp as a radiation source, absorbance mode as Scan mode, Slit dimensions of 6mm×0.6mm,scanning speed of 20mm/S, output: Densitogram and integrated peak area and measurement wavelength at 242nm.

Procedures

A) Standard solutions preparations

-Standard stock solutions of TCL and MOX were prepared in a concentration 10mg /mL in methanol.

Working standard solutions for HPLC: 1000 µg /mL of TCL and 500 µg/ mL of MOX were prepared from stock solutions by appropriate dilution with mobile phase.

Construction of calibration curve: For calibration curve construction suitable aliquots of TCL and MOX were accurately transferred from their respective working standard solutions into three separate series of 10mL volumetric flasks then completed to the volume with mobile phase to prepare 1-200 µg /mL TCL and 0.5-100 µg/ mL MOX. A volume of 20 µL was injected in triplicates in the chromatographic system under the previously mentioned chromatographic conditions and the average peak areas obtained were plotted versus corresponding concentrations and the regression equations were computed.

Working standard solutions for HPTLC: 5 mg/ mL and 1 mg/ mL solutions for TCL and MOX respectively were prepared from stock solutions by appropriate dilution with methanol. For calibration curve construction suitable aliquots of TCL and MOX were accurately transferred from their respective working standard solutions into 10 mL volumetric flasks

then completed to the volume with methanol to prepare 100-4000 µg /mL and 20-400 µg/ mL for TCL and MOX respectively. A volume of 5 µL (equivalent to 0.5-20 µg TCL and 0.1-2 µg MOX) were applied as a 5mm band in triplicates onto the HPTLC plates under the previously mentioned chromatographic conditions and the average peak areas obtained were plotted versus corresponding concentrations and the regression equations were computed.

B) Sample solutions preparation

For HPLC

Stock sample solution

An aliquot of 5mL is transferred accurately to a 100mL volumetric flask. The volume is completed to the mark with methanol (HPLC grade).

Working sample solution

Three mL aliquot of stock sample solution is transferred into three 50mL volumetric flasks, and the volume is completed to the mark with mobile phase and analyzed as described.

For HPTLC

Stock sample solution

An aliquot of 5 mL is transferred accurately to a 50 mL volumetric flask, and the volume is completed to the mark with methanol (analytical grade).

Working sample solution

A Ten mL aliquot of stock sample solution is transferred into a 25mL volumetric flask and three replicates of 5µL are applied to a HPTLC plate as triplicate using the previously mentioned auto sampler and analyzed under the previously mentioned chromatographic conditions, the average peak areas obtained were used to calculate the corresponding concentration by computing it from the regression equations.

RESULTS AND DISCUSSION

The main target for this work were the development and validation of two simple, rapid and precise chromatographic methods for the routine simultaneous quantitative determination of TCL and MOX in their combined dosage forms by high performance liquid chromatography and HPTLC densitometric method with no interference from each other or from reported excipients as none of the reported methods has fulfilled that target.

HPLC Method

The difference in polarity between TCL and MOX and the high ratio between them in the pharmaceutical dosage form (50:1) and maximum absorption wavelength of each drug were the guiding rules in selecting the type of column stationary phase, the mobile phase composition and the measurement wavelength. So a Hypersil BDS phenyl C18 column (250 × 4.6 mm, 5 µm particle size) was selected as a stationary phase due to its medium polarity and different mobile phases of different composition were tried for separation. The initial separation was developed using acetonitrile: water (50:50) but poor symmetric peaks were observed. By decreasing the pH to 2.8 using acetic acid, TCL was retained on column, while increasing the pH to up to 9 using triethylamine 0.5% the peaks were separated but poor peak symmetry occurred; finally the use of acetonitrile: methanol: 5 mM ammonium dihydrogen phosphate buffer pH 6 (60:30:10, by volume) as a mobile phase resulted in a satisfactory separation and peak symmetry with flow rate of 1 ml /min. The selection of measurement wavelength was of great importance as the ratio of TCL to MOX is 50 to 1 so maximum absorbance wavelength of MOX was selected for the determination which provided accurate and precise results. A satisfactory separation for the two drugs within less than five minutes was obtained using these optimum conditions, where the retention time values of TCL and MOX were 1.9 min and 3.9 min respectively (Figure 2). The method was subjected to the validation scheme according to ICH guidelines.[10] and good results were obtained.

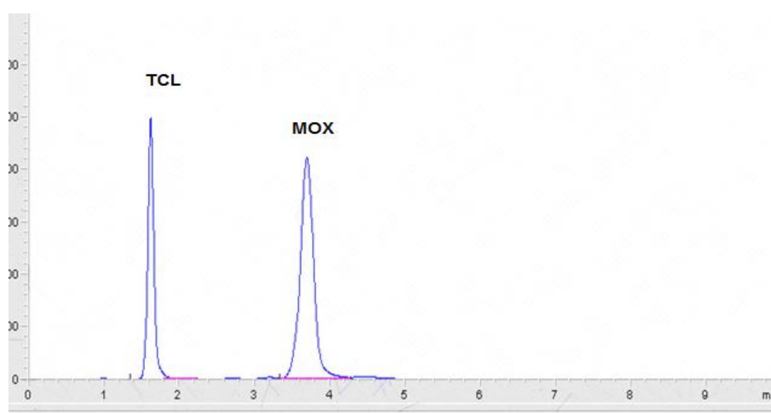

Figure 2 : HPLC chromatogram of a mixture of TCL and MOX at Rt 1.9 and 3.9 min respectively

Method validation

Specificity

It was ascertained by analyzing different laboratory mixtures containing TCL and MOX in the presence of pharmaceutical excipients and comparing retention time and area to those of

certified standard solutions. Satisfactory results were obtained indicating the high selectivity of the proposed methods. Recovery of TCL and MOX in laboratory prepared mixtures containing dosage form was calculated to express specificity (Table 1).

Linearity and range

Under the specified experimental conditions a linear relationship between peak areas and the corresponding concentrations of the drugs at the selected wavelength in the range of 1-200 µg/mL and for TCL and MOX respectively (Table 1). The proposed method was advantageous to the literature spectroscopic method for determination of MOX in bulk or in dosage forms (linearity range 8-22 µg/mL).

The regression equations were computed and found to be:

$P_{TCL}=51.39\ C+45.32$ $r=0.9995$

$P_{MOX}=45.00\ C+1.127$ $r=0.9995$

Where P is the peak area, C is the concentration in µg/mL**,** r is the correlation coefficient.

Precision

The precision of the method 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 (Table1).

Robustness

It was also checked by investigating the effect of small deliberate changes in the experimental conditions on the retention time of separated peaks. Mixtures of TCL and MOX were separated under different conditions by using different pH values 6.0 ± 1, different flow rates (1.0 ±0.5 mL/ min) and different acetonitrile composition by 60 ± 5% as 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 (Table 1).

Limit of detection and limit of quantitation

LOD and LOQ are assessed to determine the sensitivity of the method. They are calculated using the following formulae [LOD = (3* noise response) and LOQ = (10* noise response).

Table 1: Analytical parameters and validation results of the determination of TCL and MOX by the HPLC method

Method parameter	TCL	MOX
Wavelength (nm)	242	242
Linearity range	1-200 µg/mL	0.5-100 µg/mL
Time of analysis (min/run)	6	
Linearity		
Intercept	45.32	1.127
Slope	51.39	45.00
Correlation coefficient (r)	0.9995	0.9995
Specificity	100.2±0.31	100.5±0.3
Precision		
(± %RSD)	±0.35	±0.12
(± %RSD)	±0.43	±0.34
LOD	0.2 µg/mL	0.06 µg/mL
LOQ	0.6 µg/mL	0.2 µg/mL

Accuracy

Method accuracy testing was very critical as the combined pharmaceutical dosage form contains a list of excipients (Benzyl alcohol, Butyl hydroxytoluene, Polysorbate 80, Sorbitan oleate, Propylene glycol and dicaprylocaprate) so it was assessed by spiking a known previously analysed sample solution with a known quantity of certified standard and calculating recovery of the added amounts of pure standard using standard addition technique (Table 2).

Table 2: Analysis of TCL and MOX in marketed formulation by HPLC and application of standard addition technique

		Standard addition				
Product	**Proposed method % recovery**	**Taken amount(µg)**	**Added amount(µg)**	***Total found(µg)**	***Standard found(µg)**	***% Recovery of added**
Cydectin Triclamox sheep oral drench® Labeled to contain 1mg MOX and 50mg TCL / 1mL		50	0	50±0.2	-	-
		50	10	60±0.22	10±0.02	100±0.20
	TCL 100±0.14	50	20	70±0.21	20±0.01	100±0.05
		50	30	80±0.25	30±0.05	100±0.16
		Mean ±RSD*				**100±0.14**
	MOX 100±0.72	1	0.0	1±0.005	-	-
		1	0.1	1.1±0.006	0.1±0.001	100±1
		1	0.2	1.2±0.006	0.2±0.001	100±0.5
		1	0.3	1.3±0.007	0.3±0.002	100±0.67
		Mean ±RSD*				**100±0.72**

*Average of three determinations.

System Suitability

After optimizing HPLC conditions like mobile phase, wavelength, flow rate and column, different parameters such as resolution, column efficiency, tailing factor and capacity factor were calculated to ascertain the suitability of the whole chromatographic system for the intended analysis (Table 3).

Table 3: System suitability testing parameters for HPLC determination

Parameter	TCL		MOX	Reference value
K'(capacity factor)	8.9		18.9	K' > 2
α(Relative retention)		2.12		> 1
Resolution		8		R> 2
Tailing factor	1.06		1	T = 1 for typical symmetric peak
N(column efficiency)	3463		8864	The higher the value, the higher the efficiency of separation
HETP	7.22×10^{-3}		2.82×10^{-3}	The smaller the value, the higher the efficiency

HPTLC method

HPTLC procedure was optimized to develop an efficient method for quantification of TCL and MOX. Different developing systems were tried to improve the separation of pure drugs. Initially polar solvents were used as mobile phase but no separation was obtained. Decreasing the polarity by addition of some nonpolar solvents such as chloroform and methylene chloride did not enhance the separation between TCL and MOX, so further decrease in polarity was tried by mixing toluene and ethyl acetate, the separation was improved partially but the Rf of both TCL and MOX were so close to each other. Finally, a very good resolution with sharp and symmetric peaks were obtained by using ethyl acetate: toluene: formic acid 85% (5:4.5: 0.5 by volume) as a mobile phase. Rf values were found to be 0.60 and 0.90 for TCL and MOX, respectively where a good separation was obtained for the binary mixture, with good Rf values without tailing of the separated bands (Figure 3).

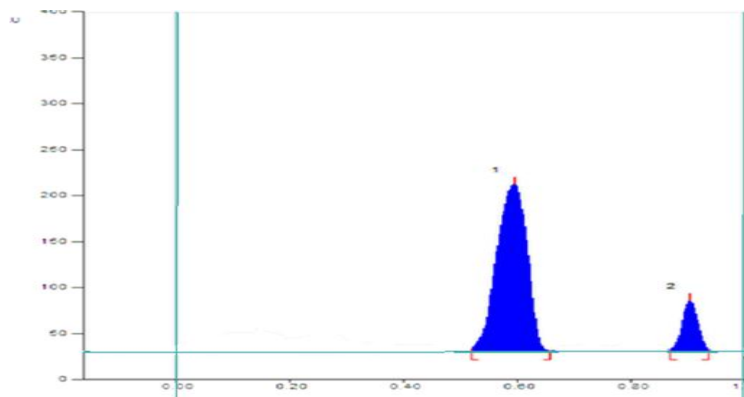

Figure 3: HPTLC Densitogram of a mixture of TCL and MOX at Rf 0.60 and 0.90 for TCL and MOX respectively

The optimum band width was 6 mm and the inter space between bands was 8.0 mm. Detection at 242 nm was suitable providing good sensitivity for TCL and MOX with minimum noise. Slit dimensions of the scanning light beam should ensure complete coverage of band dimensions on the scanned track without interference of adjacent bands 6 mm × 0.6 mm proved to be the slit dimension of choice which provides highest sensitivity. The method was subjected to the validation scheme according to ICH guidelines[10], where good results were obtained.

Method validation

Specificity

It was ascertained by analyzing different laboratory mixtures containing TCL and MOX 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 TCL and MOX in laboratory prepared mixtures containing dosage form was calculated to express specificity (Table 4).

Linearity and range

Under the specified experimental conditions, the relationships between concentrations of selected drugs and peak areas of the bands were investigated and found to linear in the range of 0.5-20 μg/band and 0.1-2 μg/band for TCL and MOX respectively. The regression equation was computed and found to be:

$P_{TCL}=6038\ C+0.329$ $r= 1$

$P_{MOX}=6466\ C+1.590$ $r=0.9995$

Where P is the peak area, C is the concentration in μg/mL**;** r is the correlation coefficient (Table 4).

Precision

The precision of the method 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 4).

Robustness

It was also checked by investigating the effect of small deliberate changes in the experimental conditions on separated spots. Mixtures of TCL and MOX were separated under different conditions by using different volumes of developing system by ±10%, different saturation times by ±20% and different ethyl acetate composition by ± 5% in the developing system. The Rf values of the separated spots using the mentioned volumes of developing system range did not change, while changing ethyl acetate composition and saturation times was accompanied by slight decrease or increase of Rf of the two peaks. This did not affect separation (Table 4).

Limit of detection and limit of quantitation

LOD and LOQ are assessed to determine the sensitivity of the method. They are calculated using the following formulae. [LOD = (3* noise response) and LOQ = (10* noise response) (Table 4).

Table 4: Analytical parameters and validation results of the determination of TCL and MOX by the HPTLC method

Method parameter	**TCL**	**MOX**
Wavelength (nm)	242	242
Linearity range	0.5-20 µg/band	0.1-2µg/band
Time of analysis (min/run)	30	
Linearity		
Intercept	0.329	1.59
Slope	6038	6466
Correlation coefficient (r)	1	0.9995
Specificity	100.0±0.01	100.0±0.05
Precision		
(± %RSD)	± 0.75	±0.62
(± %RSD)	±0.81	±0.73
LOD	0.05µg/band	0.035 µg/band
LOQ	0.20 µg/band	0.08 µg/band

Accuracy

Method accuracy was assessed by spiking a known previously analysed sample solution a known quantity of certified standard and calculating recovery of the added amounts of pure standard using standard addition technique (Table 5).

Table 5: Analysis of TCL and MOX in marketed formulation by HPTLC densitometry and application of standard addition technique

Product	Standard addition					
	Proposed method % recovery	Taken Amount (µg)	Added Amount (µg)	*Total Found (µg)	*Standard Found (µg)	*% Recovery of added
Cydectin Triclamox sheep oral drench® Labeled to contain 1mg MOX and 50mg TCL / 1mL	TCL 99.66±0.66	7.5	0	7.5±0.04	-	-
		7.5	5	12.5±0.09	5±0.05	100±1
		7.5	7.5	14.94±0.10	7.44±0.008	99.2±0.106
		7.5	9	16.48±0.12	8.98±0.08	99.77±0.88
		Mean ±RSD*				**99.66±0.66**
	MOX 99.64±0.62	0.15	0.0	0.15±0.003	-	-
		0.15	0.1	0.249±0.005	0.0996±0.002	99.6±0.02
		0.15	0.15	0.299±0.005	0.149±0.0004	99.33±1.33
		0.15	0.2	0.35±0.004	0.201±0.0003	100 ±0. 5
		Mean ±RSD*				**99.64±0.62**

*Average of three determinations.

System Suitability

After optimizing HPTLC conditions like developing system composition, wavelength, saturation time, and different parameters such as resolution, relative retention, symmetry factor and capacity factor were calculated to ascertain the suitability of the whole chromatographic system for the intended analysis (Table 6).

Table 6: System suitability testing parameters for HPTLC determination

Parameter	TCL		MOX
K' (capacity factor)	2.33		3.66
α(Relative retention)		1.6	
Resolution		13.3	
Symmetry factor	0.99		1

CONCLUSION

The accuracy, simplicity, time and cost effectiveness of the proposed methods, confirm their suitability for use as routine quality control methods for both drugs in their combined dosage forms without prior separation or interference from reported excipients.

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