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Validated TLC-Densitometric Method for Determination of Amprolium Hydrochloride and Ethopabate in Veterinary Preparation

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Abstract

A simple precise accurate TLC- densitometric method was presented for the quantitative determination of amprolium hydrochloride and ethopabate in combined veterinary preparations and in presence of Levamisole hydrochloride. The compounds were developed on TLC aluminum plates pre-coated with silica gel 60 F254 using methanol: water: 0.1% acetic acid (7:2.5:0.5, v/v/v). The plates were air dried, detected under UV lamp and then scanned at 213 nm. The validity of the proposed method was assessed using the standard addition technique. The obtained results were statistically compared with those obtained by the official method, showing no significant difference with respect to accuracy and precision at p=0.05.

Keywords: Amprolium hydrochloride; Ethopabate; Levamisole hydrochloride; TLC-densitometry

Introduction

Coccidiosis is a term sometimes applied to infections with protozoa of the order Eucoccidiorida. The predominant coccidian infections in man are caused by Cryptosporidium, Cyclospora cayetanensis, Isospora, Plasmodium, and Toxoplasma. Coccidian protozoa, primarily Eimeria, cause economically important infections in domesticated animals [1]. Amprolium hydrochloride and Ethopabate are widely used to treat and prevent coccidiosis in chickens. Since both are usually used as a combination, it is important to develop simple spectrophotometric methods to determine them simultaneously. Amprolium hydrochloride is 1-[(4-amino-2-propyl-5-pyrimidinyl) methyl]-2-methylpyridinium chloride hydrochloride [2] (Figure 1). It is an antiprotozoal used in veterinary practice alone or with other drugs such as Ethopabate, for the control of coccidiosis in pigeons and in poultry [1]. Ethopabate is methyl 4-acetamido-2-ethoxybenzoate [2] (Figure 2). It is an antiprotozoal used in veterinary practice with other drugs, such as amprolium, for the control of coccidiosis in poultry [1]. Literature survey revealed that Amprolium hydrochloride and Ethopabate are official in British Pharmacopoeia [3]. There are many reported methods for the determination of either AMP, ETH, together or in combination with other drugs in different matrices such as pharmaceutical formulation, surface water, eggs, chicken muscles, chicken plasma, chicken liver and chicken feed. These

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methods include liquid chromatography coupled with ultraviolet (UV) [4,5] or fluorescence [6-9] detection, and liquid chromatography mass spectrometry (LC–MS) [10-16], Thin layer chromatography [4], Spectrophotometric methods [17-21] Atomic spectrometry[22], Capillary electrophoresis [23], Electrochemical method [24].

Chickens can be infected with roundworms and coccidia. Heavy infections cause weight loss, diarrhoea and poor egg production [25]. So, it is a common practice to administer an anthelmintic with anticoccidial drugs to treat helminthiasis and coccidiosis. Levamisole hydrochloride (Figure 3**)** (LEV) is widely used as an anthelmintic in cattle, sheep, goats, swine, and poultry. It is effective against gastrointestinal nematodes [26].

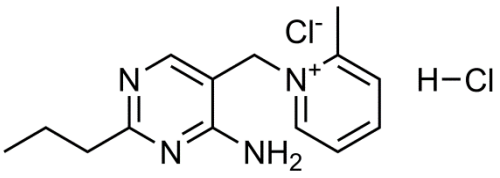

Figure 1: Structure of amprolium hydrochloride.

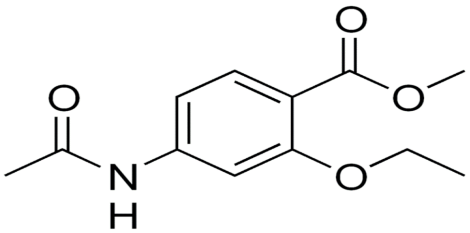

Figure 2: Structure of Ethopabate.

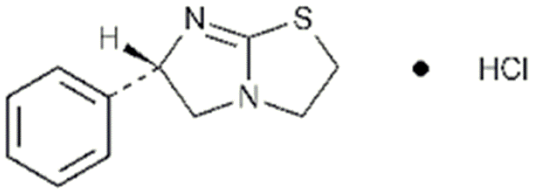

Figure 3: Structure of Levamisole hydrochloride.

The literature survey revealed that no methods have been reported for the analysis of AMP and ETH in combination with the co-administered drug LEV. The aim of the present work is to develop a new, simple, accurate and precise TLC-densitometric method validated according to ICH guidelines.

Experimental

Instrumentation

Desaga densitometer model CD 60 (Germany)

AS 30 Desaga applicator

Desaga UV lamp with short wavelength (254 nm)

TLC aluminium plates pre-coated with silica gel 60 F_{254} (E. Merck).

Chromatographic tank 20 x 21 x 9 cm

Chemicals and reagents

Amprolium hydrochloride and Ethopabate are kindly supplied by Prima Vet pharmaceutical company, Cairo, Egypt. Their purity is found to be 100.35% and 99.5%, respectively, according to the reported spectrophotometric method [26].

Levamisole hydrochloride (LEV) kindly supplied by Amoun pharmaceutical company, Obour city, Qhalubeya, Egypt. Its purity was certified to be 100.20%.

All chemicals and solvents used were of analytical grade:
Methanol *(Riedel-de Haën, Sigma-Aldrich, Germany)*
Bi-distilled water and is indicated by "water".
Glacial acetic acid (E. Merck, Darmastadt, Germany) was used to prepare 0.1% acetic acid

Standard solutions

A stock solution of AMP was prepared by dissolving 0.1 gm of AMP in 100 mL methanol. A stock solution of ETH was prepared by dissolving 0.1 gm of ETH in 100 mL methanol. Similarly, a stock solution of LEV was prepared by dissolving 0.1 gm of LEV in 100 mL methanol.

Procedure

Chromatographic conditions: Analysis was performed on 20 × 20 cm TLC aluminum plates pre-coated with silica gel 60 F_{254} (E.Merck). Spots were applied 1 cm apart from each other and 2 cm apart from the bottom edge. The chromatographic tank was pre-saturated with the developing system for 30 min at room temperature. The plates were developed by ascending chromatography for 8 cm, using methanol: water: 0.1% acetic acid (7:2.5:0.5, v/v/v) as a developing system. The plates were air dried, detected under UV lamp and then scanned at 213 nm under the following experimental conditions of measurement:
Photo mode: Reflectance.
Scan mode: Linear slit scanning.
Slit width=0.4 mm.
Slit height=0.02 mm.
Result output: Densitogram and peak list.

Method validation

Linearity: Different aliquots equivalent to (0.4 - 10 µg/spot) of AMP, (0.4 - 19 µg/spot) of ETH and (0.4 - 10 µg/spot) of LEV were applied to the TLC plates, in triplicates, the chromatographic conditions were adjusted, the plates were developed and scanned at 213 nm. Peak areas were measured and calibration curves relating the peak areas and their corresponding concentrations for each drug were constructed and the regression equations were then computed.

Accuracy: The previously mentioned procedure under linearity was repeated for determination of different concentrations of pure samples of AMP, ETH and LEV in triplicates. The concentrations were calculated from the corresponding regression equations and the mean recovery percentages and standard deviations were then calculated.

Precision: The intraday and interday variations were evaluated by applying the previously mentioned procedure, for analysis of 5, 7 and 9 µg/spot of AMP, 5, 12 and 14 µg/spot of ETH and 1, 3 and 5 µg/spot of LEV each three times (n=9) on the same day and on three successive days, respectively. The concentrations were calculated from the corresponding regression equation, the recovery percentages and standard deviations were then calculated.

Specificity: The specificity of the method was established through studying the resolution (Rs) for each drug from the nearest resolving peak, also the selectivity factor (α) was studied.

Limit of detection (LOD) and limit of quantification (LOQ): LOD and LOQ were calculated using the corresponding calibration curve. According to the ICH guidelines for determination of LOD and LOQ, the estimation was based on the standard deviation of response**.**

$LOD = 3.3 \times \sigma / S$

$LOQ = 10 \times \sigma / S$

Where, σ is the standard deviation of response and S is the slope of the calibration curve. Here, the standard deviation of the y-intercept of the regression line can be used as the standard deviation of response.

Application of the proposed method for the determination of AMP and ETH in pharmaceutical formulation (Amprolium & Ethopabate premix 25%®): For determination of AMP, 0.1 gm of the premix equivalent to 25 mg of AMP was accurately weighed, sonicated in 25 mL methanol for 15 min and filtered into 100- mL volumetric flask. The residue was washed three times each with 5 mL methanol and then completed to volume with methanol.

For determination of ETH, 1 gm of the premix equivalent to 16 mg of ETH was accurately weighed, sonicated in 25 mL methanol for 15 min and filtered into 100- mL volumetric flask. The residue was washed three times each with 5 mL methanol and then completed to volume with methanol.

The previously mentioned procedure under linearity was repeated and the concentrations of AMP and ETH were calculated from the corresponding regression equations and the mean recovery percentages and standard deviations were then calculated.

Standard addition technique was applied by analyzing the pharmaceutical formulation spiked with different concentrations of pure standard drug. These concentrations of AMP and ETH were calculated from the corresponding regression equations and the mean recovery percentages and standard deviations were then calculated.

Results and Discussion

TLC-densitometric technique was proposed for simultaneous determination of AMP and ETH in presence of LEV, an anthelmintic commonly co-administered with anticoccidial drugs to treat helminthiasis and coccidiosis in chickens. The technique was based on the difference in R_f values of AMP, ETH and LEV. Mixtures of AMP, ETH and LEV were spotted separately at equivalent locations on TLC plates. The plates were developed in the traditional linear ascending manner for 8 cm, where the best resolution was obtained. The plates were air dried and the separated drugs` spots were determined densitometrically on the plates at 213 nm.

Experimental conditions such as developing system and wavelength of detection were optimized to provide accurate, precise and reproducible results. Different developing systems were tried such as methanol: water (7: 1, v/v). With this developing system, the spots of AMP and LEV remained at the baseline due to their hydrophilic nature as both are hydrochloride salts. Thus, the polarity of this system was increased by adding more water and different volumes of 0.1% acetic acid. The best resolution of the three drugs was achieved by using methanol: water: 0.1% acetic acid (7:2.5:0.5, v/v/v). The R_f values were 0.1, 0.34 and 0.83 for AMP, LEV and ETH, respectively. (Figure 4)

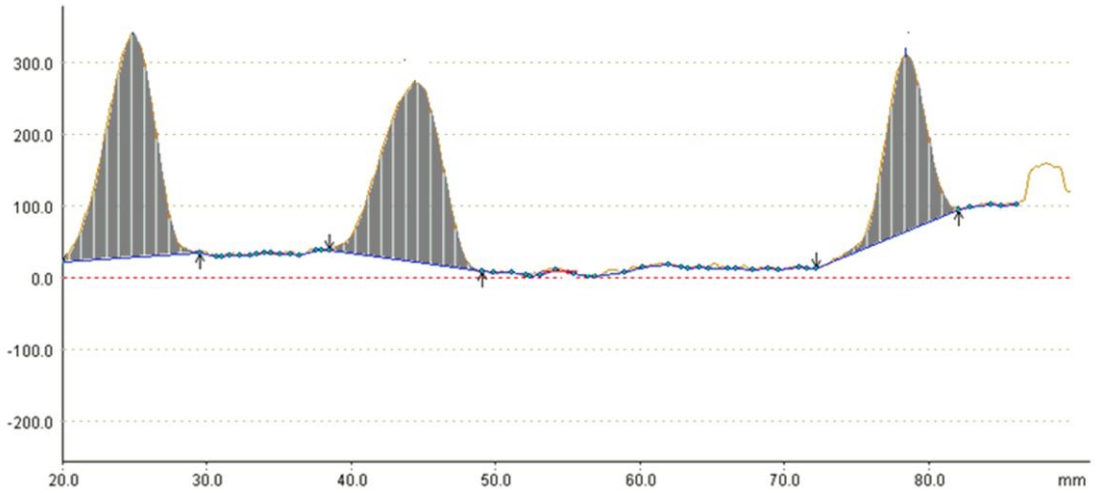

Figure 4: TLC Densitogram showing excellent separation of AMP (Rf=0.1), LEV (Rf=0.34) and ETH (Rf=0.83).

The calibration curves in TLC are generally inherently non-linear due to scattering of light. They generally comprise a pseudo-linear region at low sample concentrations and then departure from linearity begins at higher sample concentrations [27]. With TLC, the analyte interacts with the layer surface of the stationary phase where scattering and absorption tend to take place specially with high concentrations of analyte [28]. These combined processes are not adequately described by Beer-Lambert`s law, but the Kubelka Munk model [29].

Moreover, the ICH guidelines mentioned that for some analytical procedures which do not demonstrate linearity, the analytical response should be described by an appropriate function of the concentration of the sample. The relationship between the integrated peak area and the concentration was evaluated with linear and polynomial regression functions. Fitting with linear function gave correlation coefficients (r) values of 0.9993, 0.9916 and 0.9972 for AMP, ETH and LEV, respectively. While, fitting with 2^{nd} order polynomial function gave better (r) value of 0.9996 for each of AMP, ETH and LEV and lower standard deviation values and was therefore used for quantitative analysis.

Calibration curve for AMP, ETH and LEV were constructed in the range of 0.4 – 10 µg/spot, 0.4 – 19 µg/spot and 0.4 – 10 µg/spot, respectively. The polynomial regression equations were computed and found to be: $A=-5.0940C^2 + 554.6252C + 0.7296$, r=0.9996 for AMP, $A=-3.6401C^2 + 195.0667C + 126.3503$, r=0.9996 for ETH and $A= -12.7586C^2 + 573.2461C - 45.237$, r=0.9996 for LEV, Where, A is the peak area, C is the concentration in µg/spot and r is the correlation coefficient.

The proposed method was successfully applied for the determination of AMP, ETH and LEV in bulk powder with mean recovery percentages of 100.72 ± 1.189, 99.93 ± 1.258 and 100.53 ± 0.654, respectively **(Table 1).**

AMP			ETH			LEV		
Taken (µg/spot)	**Found (µg/spot)**	**% Recovery**	**Taken (µg/spot)**	**Found (µg/spot)**	**% Recovery**	**Taken (µg/spot)**	**Found (µg/spot)**	**% Recovery**
0.9	0.900	100.00	0.4	0.391	97.75	0.9	0.905	100.56
3	3.084	102.80	5	5.046	100.92	1	0.998	99.80
5	5.001	100.02	6	6.035	100.58	3	3.047	101.57
7	7.011	100.16	12	12.010	100.08	5	5.011	100.22
9	9.057	100.63	14	14.045	100.32	7	7.034	100.49
Mean ± S.D.		100.72 ± 1.189	**Mean ± S.D.**		99.93 ± 1.258	**Mean ± S.D.**		100.53 ± 0.654
R.S.D%		1.181	**R.S.D%**		1.259	**R.S.D%**		0.651

* Average of three determinations

Table 1: Results of accuracy for the determination of pure samples of AMP, ETH and LEV by the proposed TLC-densitometric method.

The specificity of the proposed method was illustrated in figure 63 where complete separation of the three drugs was shown. Consequently the results of system suitability tests assured that AMP, ETH and the commonly co-administered anthelmintic LEV can be determined simultaneously without interference from each other **(Table 2).**

Parameter	**AMP**	**ETH**	**LEV**	**Reference value**
Retention factor (R_f)	0.1	0.83	0.34	-
Selectivity factor (α)	4.64	9.7	4.64	**α** >1
Resolution (R_s)	2.5	4.74	2.5	$\mathbf{R_s}$ >1

Table 2: System suitability test results of the proposed TLC-densitometric method for the determination of AMP, ETH and LEV.

The proposed method was successfully applied for determination of AMP and ETH in their combined pharmaceutical formulation and its validity was assessed by applying the standard addition technique (**Table 3).** The results of analysis of the pharmaceutical formulation and the standard addition technique suggested that there was no interference from any excipients.

Dosage form	Drug	TLC-densitometric method					
Amprolium & Ethopabate premix 25%® (250 gm AMP and 16 gm ETH/ Kg)	**AMP**	**Taken (µg/spot)**	**Found (µg/spot)**	**% recovery***	**Added (µg/spot)**	**Found (µg/spot)**	**% recovery***
		2.5	2.526	101.04	4	3.996	99.90
					5	5.057	101.14
					6	6.018	100.30
	Mean ± S.D.						100.45 ± 0.633
	ETH	1.6	1.637	102.31	4	3.998	99.95
					5	5.002	100.04
					6	5.995	99.92
	Mean ± S.D.						99.97 ± 0.062

*average of three determinations

Table 3: Determination of Amprolium hydrochloride and Ethopabate in their pharmaceutical formulation by the proposed TLC-densitometric method and application of standard addition technique.

Statistical comparison showed that there was no significant difference between the results obtained from the proposed method and those obtained from the reported method. The proposed method was found to be accurate and precise since the t and F values were less than the tabulated ones **(Table 4).**

Value	TLC-densitometric method		Reported method[b]	
	AMP	**ETH**	**AMP**	**ETH**
Mean	100.72	99.93	100.35	99.50
SD	1.189	1.258	1.560	1.543
RSD%	1.181	1.259	1.555	1.551
N	5	5	6	6
Variance	1.414	1.583	2.434	2.381
Student`s t-test[a]	0.437 (2.262)	0.497 (2.262)	_	_
F value[a]	1.720 (6.256)	1.504 (6.256)	_	_

[a] The values in parenthesis are the corresponding theoretical values of t and F at (P=0.05)

[b] First derivative spectrophotometry at 288.8 and 320.6 nm for AMP and ETH, respectively

Table 4: Statistical comparison of the results obtained by applying the proposed TLC-densitometric method and the reported method for the analysis of pure Amprolium hydrochloride and Ethopabate.

Validation of the proposed method for the simultaneous determination of AMP, ETH and LEV was made by measuring concentration range, linearity, accuracy, specificity, precision and limits of detection and quantification, according to ICH guidelines **(Table 5).**

Parameters	**TLC-densitometric method**		
	AMP	**ETH**	**LEV**
λ (nm)	213	213	213
Concentration range (µg/spot)	0.4 – 10	0.4 – 19	0.4 – 10
Linearity			
Slope	554.6252	195.0667	573.2461
Intercept	0.7296	126.3503	-45.2376
Correlation coefficient (r)	0.9996	0.9996	0.9996
Accuracy (mean ± S.D.)	100.72 ± 1.189	99.93 ± 1.258	100.53 ± 0.654
Specificity	specific	specific	specific
Precision (%RSD)			
Repeatability[a]	0.297	0.958	0.739
Intermediate precision[b]	0.227	1.122	0.678
LOD[c] (µg/spot)	0.061	0.038	0.075
LOQ[c] (µg/spot)	0.184	0.116	0.228

[a] The intraday (n=3), average of three different concentrations repeated three times within day.

[b] The interday (n=3), average of three different concentrations repeated three times in three successive days.

[c] Limit of detection and limit of quantification

Table 5: Assay parameters and method validation for the determination of pure samples of AMP, ETH and LEV by the proposed TLC-densitometric method.

The proposed method show better sensitivity, better LOD and LOQ values than a reported TLC method [4] **(Table 6).** Besides, our accuracy, specificity and precision values are within acceptable ranges.

Parameters	**Proposed TLC densitometric method**		**Reported TLC densitometric method**	
	AMP	**ETH**	**AMP**	**ETH**
Concentration range (µg/spot)	0.4 – 10	0.4 – 19	2-25	1-10
Accuracy (mean ± S.D.)	100.72 ± 1.189	99.93 ± 1.258	99.80 ± 1.058	99.37 ± 0.954
Precision (%RSD)				
Repeatability	0.297	0.958	0.780	0.88
Intermediate precision	0.227	1.122	1.030	0.11
LOD (µg/spot)	0.061	0.038	0.4	0.05
LOQ (µg/spot)	0.184	0.116	1.32	0.15

Table 6: Comparison of the results obtained by the proposed TLC densitometric method and the reported TLC method.

Conclusion

The proposed method was simple, rapid, sensitive, accurate and capable of full resolution of the two anticoccidial drugs amprolium hydrochloride and ethopabate in presence of the commonly co-administered anthelmintic drug levamisole hydrochloride. Also, the proposed method was suitable for routine quality control analysis of amprolium hydrochloride and ethopabate in their pharmaceutical formulation.

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