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Development and Validation of RP-HPLC Method for Simultaneous Determination of Amprolium HCl and Ethopabate in Their Combination Drug

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Abstract: In this study a simple, rapid, accurate, sensitive and specific reverse phase-high performance liquid chromatographic (RP- HPLC) method was developed and subsequently validated for simultaneous estimation of Amprolium hydrochloride (AMP) and Ethopabate (ETH) in their combination syrup. The separation of the drugs was carried out using a base deactivated silanol (BDS) C18 (250mm x 4.6mm, 5 µm) column, mobile phase consisting of methanol and purified water in the proportion of 60:40 (v/v) containing 0.5% Heptansulfonic acid sodium at pH of 3.7 and flow rate of 1 ml/min. The influence of the instrument operating conditions on the resolution and retention time were tested. The method was linear over a range of 48-480 µg/ml and 3-30 µg/ml with a correlation coefficient (r2) of 0.99996 for AMP and ETH, respectively. The method validations study revealed excellent accuracy, precision, linearity, specificity, limit of detection (LOD) and limit of quantitation (LOQ) of the proposed method according to the international conference harmonization (ICH) guidelines. Moreover, the stability study revealed that the proposed method can also be used for evaluation of purity and degradation of these drugs in their formulations that arisen due to the temperature, humidity and time.

Keywords: Amprolium HCl, Ethopabate, Validation, Combination, HPLC

1. Introduction

During the past decades a variety of efforts have been focused to control the coccidiosis through sanitation, chemotherapy, immunogenic and nutrition methods [1]. However, anticoccidial drugs have been used as prophylactic or therapeutic agents in chickens [2-7]. Amprolium hydrochloride (AMP) which is 1-[(4-amino-2-propyl-5-pyrimidinyl) methyl]-2-methylpyridinium chloride hydrochloride [8, 9] and Ethopabate (ETH) which is methyl 4-acetamido-2- ethoxybenzoate, are widely used as anticoccidial drugs [9, 10]. Since both are usually used as a combination drug, it is important to develop simple analytical method to determine them simultaneously. Many analytical methods such as electrochemical [11], liquid chromatography–mass spectrometry (LC–MS) [12-18], spectrophotometric [19-23], spectrofluorimetric [24], potentiometric [25], capillary electrophoresis [26], thin layer chromatography [27] and atomic spectrometry [28] methods were reported for the determination of AMP and ETH in different matrices. Nevertheless, these methods offer a high grade of specificity, but still they are associated with some drawbacks such as sample preparation, time consuming to reach equilibration and/or require the use of large quantities of chemical reagents. Therefore, there is a need to develop a fast, specific, and accurate method that allows the simultaneous determination of the tow active ingredients within a reasonable retention time.

HPLC-based methods are recognized as highly sensitive methods for isolating and determining analysts in different matrices. In addition, they are the most extensive analytical method that has been developed for simultaneous determination of combined drugs in different matrices [29-32]. Therefore, in this study, we developed and validated a

simple, rapid, accurate, sensitive and specific RP-HPLC method for the simultaneous determination of AMB and ETB in their twofold mixtures.

2. Experimental

2.1. Chemicals

Amprolium HCl working standard (98.2% pure) was obtained from Aurum Research Centre (Amman, Jordan). Ethopabate working standard (97.2% pure) was obtained from India Pharma. Methanol (HPLC Grade) & Glacial Acetic Acid (Analytical Grade) from CARLO ERBA Reagents (Italy). Heptansulfonic acid Sodium.

2.2. Instrumentation

HPLC system containing a stainless steel column (BDS C 18,250mm x 4.6mm 5.0mμ) mentioned at ambient temperature, with analytical wavelength set at 262 nm.

2.3. Preparation of Calibration Curves

Standard solutions of AMP and ETH (2400 μg/ml and 150 μg/ml, respectively) were used to prepare serial dilutions in methanol: water (70:30) in the ranges of 48-480 μg/ml and 3-30 μg/ml of AMP and ETH, respectively.

2.4. Preparation of Test Solution

Fortified test solution was prepared using standard solutions of AMP and ETH (2400 μg/ml and 150 μg/ml, respectively) mixed with 1 ml of Super Amprol formulation in 100 ml volumetric flask, and made up to the mark using methanol. Subsequently, three fortified samples were prepared in the ranges of 120-360 μg/ml and 15-22.5 μg/ml of AMP and ETH, respectively. Afterwards, the spiked solutions were shook well, and filtered through 0.45μl nylon filters and injected into the HPLC system.

2.5. Method Validation

The method was validated according to the United States Pharmacopeia (USP), International Conference on Harmonization (ICH), and the Food and Drug Administration (FDA) [33-35]

2.5.1. Linearity

For the linearity study, stock solution was prepared as in the previous section. A series of nine concentration levels in the ranges of 48-480 μg/ml and 3-30 μg/ml of AMP and ETH, respectively.

2.5.2. Specificity

The specificity of the method was evaluated via testing peaks purities of AMP and ETH. Moreover, the specificity was measured in relation to mobile phase, diluted standard of AMP and ETH, and the placebo formulation. Then injected into the HPLC system to detect the possible interfering peaks.

2.5.3. Accuracy

The fortified sample was prepared by standard addition in a placebo formulation as in the test solution. The spiked solutions were prepared in triplicate for each fortified sample and the recoveries were calculated.

2.5.4. Precision

The intra-day precision of the method was evaluated by assaying of six determinations (n = 6) at 100% of the test concentration (240 μg/ml and 15 μg/ml of AMP and ETH, respectively) during the same day. Evaluation of the inter-day precision was carried out on successive days (n = 3). The precision results were calculated and stated as relative standard deviation (RSD %).

2.5.5. Robustness

The robustness of the method was checked by varying the instrumental conditions such as flow rate, Organic content in mobile phase ratio, wavelength of detection and column temperature through injecting triplicate injections of the standard solutions, and assaying of three determinations at 100% of the test concentrations of the same Super Amprol Batch used in the precision Study.

3. Results and Discussions

3.1. Optimization of the HPLC Conditions and Stability Study

In order to find the best retention time and resolution between the AMP and ETH peaks, experiments were carried out via varying the mobile phase conditions and the flow rate using standard solutions of AMP and ETH. The best resolution was found using a mixture of methanol: water (60:40) containing 0.5% of heptansulfonic acid sodium, and the pH was adjusted to be 3.7 using glacial acetic acid as a mobile phase after filtering and degassing for 10 min. The optimum flow rate was found to be 1 ml/min.

The system suitability test was achieved from five replicate injections of standard working solution (240 μg/ml and 15 μg/ml of AMP and ETH, respectively). As seen in tables 1 and 2, the RSD values for the tested parameters were less than 2, which confirmed that the HPLC system has excellent stability for both drugs.

Table 1. *Result of System suitability test of AMP.*

	Parameters			
Injection	**Ret. Time**	**Peak Area**	**Theo. Plate**	**Tailing Factor**
1	11.742	10462949	10672.37	1.243
2	11.565	10477581	10685.61	1.243
3	11.614	10468155	10702.17	1.242
4	11.581	10455528	10740.38	1.24
5	11.546	10453760	10729.8	1.24
Average	11.6096	10463595	10706.07	1.2416
STDEV	0.078104	9729.116	28.76507	0.001517
RSD	0.672757	0.092981	0.26868	0.122147

Table 2. *Result of System suitability test of Ethopabate.*

	Parameters			
Injection	**Ret. Time**	**Peak Area**	**Theo. Plate**	**Tailing Factor**
1	7.166	1304516	9504.924	1.089
2	7.137	1304139	9526.516	1.091
3	7.124	1304799	9511.913	1.093
4	7.113	1302604	9545.557	1.092
5	7.101	1302746	9533.59	1.094
Average	7.1282	1303761	9524.5	1.0918
STDEV	0.024974	1019.709	16.36874	0.001924
RSD	0.350355	0.078213	0.171859	0.17618

3.2. Calibration Curves

The calibration curves were obtained by plotting the concentrations of AMP and ETH standards (48-480 µg/ml and 3-30 µg/ml of AMP and ETH, respectively) versus their corresponding peak areas (obtained by HPLC). As in fig 1(a & b), the calibration curves were linear in the ranges of the tested concentrations.

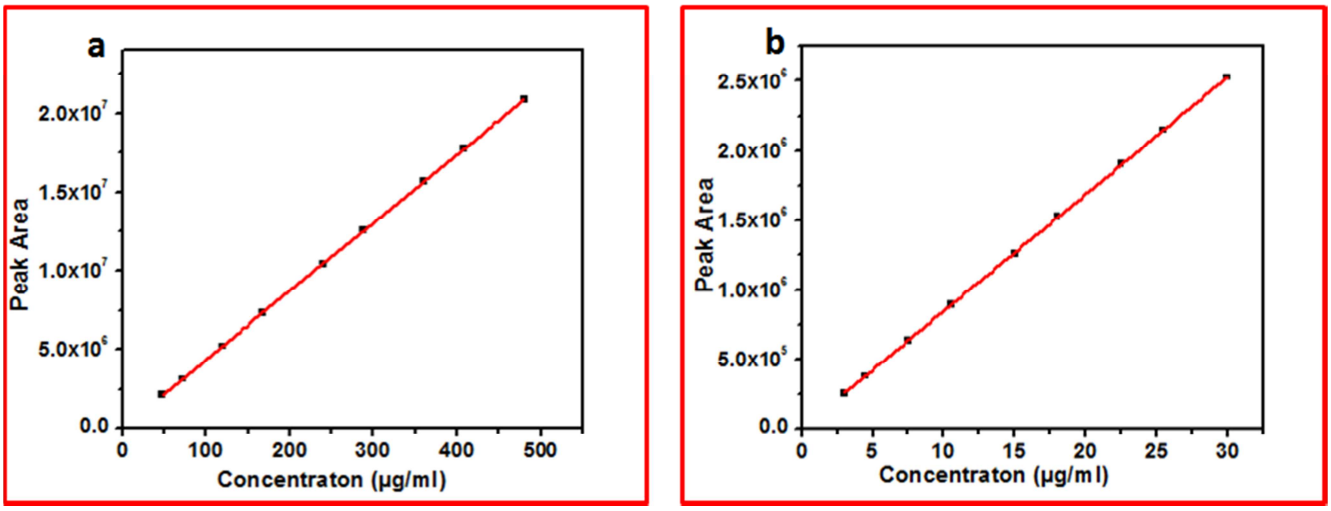

Figure 1. *Calibration curves of (a) AMP; (b) ETH.*

3.3. Method Validation

In this study the analytical method was developed to provide a fast, accurate and efficient determination of AMP and ETH in Super Amprol syrup. The developed method was validated by means of linearity, limit of detection (LOD), limit of quantitation (LOQ), specificity, accuracy, precision and robustness.

3.3.1. Linearity, LOD and LOQ

The linearity of the HPLC method was computed by regression analysis using the calibration data, and the values of regression coefficient (r2), LOD and LOQ were shown in table 3. The LOD and LOQ for both AMP and ETH were calculated using the expressions:

$$LOD = 3.3*SD/S \quad (1)$$

$$LOQ = 10*SD/S \quad (2)$$

Where SD is the standard deviation of the y-intercepts of the regression line, and S is the slope of the calibration curve.

As seen in fig 1(a and b) the method was linear in the ranges of 48-480 µg/ml and 3-30 µg/ml of AMP and ETH, respectively. The LOD and LOQ were found to be 3.002 and 9.098 µg/ml, and 0.210 and 0.637 µg/ml for AMP and ETH, respectively.

Table 3. *Linearity, LODs, LOQs and recoveries of AMP and ETH in spiked sample.*

Drugs	**Linear range (µg/ml)**	**R2**	**LOD (µg/ml)**	**LOQ (µg/ml)**	**Recoveries %**
AMP	48-480	0.99996	3.002	9.098	99.47 ± 0.24
ETH	3-30	0.99996	0.210	0.637	98.94 ± 0.28

3.3.2. Specificity

Specificity is the ability of a method to discriminate between the analyst (s) of interest and other components that are present in the sample. The method was shown no interference from placebo at the retention time of the drugs peaks, fig 2 (a, b, c and d).

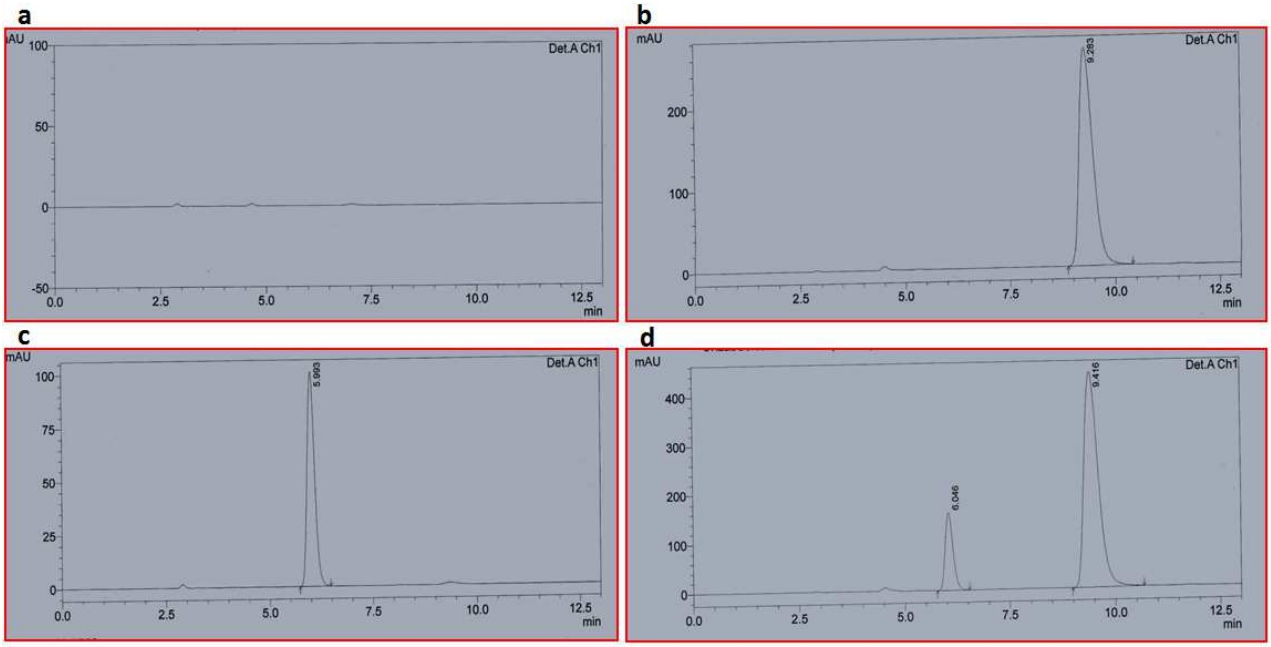

Figure 2. *Chromatograms of (a) placebo; (b) standard solution of AMP; (c) standard solution of ETH; (d) combined drug sample (AMB + ETH).*

3.3.3. Accuracy

Accuracy is the closeness between the accepted true value or a reference value and the actual result obtained. Accuracy studies are usually evaluated by determining the recovery of a spiked sample of the analyst into the matrix of the sample to be analyzed. The accuracy of the method was evaluated by determination of the recoveries of three concentrations covering the range of the method. The amount of AMP and ETH were recovered in the presence of placebo interference. As clearly seen in table 3, the mean recovery of AMP and ETH were calculated to be 99.47 ± 0.24% and 98.92 ± 0.28%, respectively. Where the RSD values were lower than 2.0%, demonstrating that the method has acceptable accuracy for the simultaneous determination of the two drugs.

3.3.4. Precision

The contents of AMP and ETH in the intra-day and inter-day precision studies are shown in table 4. The RSD% values of intra-day precision were 0.91% and 0.64% for AMP and ETH, respectively. The % RSD values for inter-day precision were 0.32% and 0.63% for AMP and ETH, respectively. As obtained, the values of RSD are lower than those for intra-day and inter-day analyses (2.0% and 5.0%, respectively). Which confirm the precision of the developed method.

Table 4. *Contents of AMP and ETH in the intra-day and inter-day precision study.*

Drugs		Intra-day precision (n = 6)	Inter-day precision (n = 3)
AMP	Contents %	101.49	101.11
	RSD %	0.91	0.32
ETH	Contents %	101.71	101.69
	RSD %	0.64	0.63

3.3.5. Robustness

The content values for each parameter changed for the drugs under study were compared with those of the original analytical method. The results were summarized in table 5. As seen, the RSD values of the tested parameters were less than 2%, which indicate that the method was robust for changes in wavelength, mobile phase flow rate and column temperature for AMP and ETH.

Table 5. *The average contents of the tested robustness parameters.*

Drugs		Wavelength (λ = 262 nm)	Flaw Rate (1 ml/min)	(Wavelength & Flow Rate)	(Column Temperature)
AMP	Contents %	102.2	102.2	102.3	101.2
	RSD %	0.57	0.11	0.10	0.06
ETH	Contents %	101.5	101.2	100.8	100.3
	RSD %	0.11	0.11	0.00	0.00

From the results and discussions, we can confirm that the developed method was successfully validated for the simultaneous determination of AMP and ETH in their combination formulations. Moreover, the stability study revealed that the proposed method can also be used for the evaluation of the purity and the stability of these drugs in their formulations that arisen due to the temperature, humidity and time. In addition, we suggest that this method can also be applied for the determination of AMP and ETH in chickens plasma, eggs and other chicken products, after sample pretreatment and cleanup steps.

4. Conclusion

In this study, we developed and validated simple, rapid, accurate, sensitive and specific RP-HPLC method for the simultaneous determination of AMP and ETH in a pharmaceutical dosage form (syrup). We believe that the method can be used for the routine analysis of AMP and ETH in their available formulation. Moreover, the developed method is valid and suitable for laboratory application using HPLC system.

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