Validated HPLC Method to Simultaneously Determine Amprolium Hydrochloride, Sulfaquinoxaline Sodium and Vitamin K₃ in A.S.K Powder on ZIC-HILIC Column

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Abstract

A new HPLC method that is based on zwitterionic hydrophilic interaction liquid chromatography (ZIC-HILIC) coupled with ultraviolet detection was developed, optimized and validated for the simultaneous determination of amprolium hydrochloride, sulfaquinoxaline sodium and Vitamin K₃ (as menadione sodium bisulfite) in A.S.K powder. The separation was carried out using ZIC-HILIC column (250 mm × 4.6 mm, 5 μm) and a mobile phase of 0.2 M Ammonium acetate (NH₄AC) buffer and acetonitrile (15:85; v/v) with pH adjusted to 5.7 by glacial acetic acid at a flow rate of 0.5 ml/min. The analytes were monitored by UV detection at 263 nm.

The effects of the operational chromatographic conditions on retention and resolution were tested. Different concentrations of the organic solvent in the mobile phase, the ionic strength of the NH₄AC buffer and pH of the mobile phase were investigated.

The optimized method was subjected to validation by examining specificity, accuracy, precision, linearity, range, ruggedness and robustness. The results were evaluated as per the International Conference on Harmonization (ICH) and United States Pharmacopoeia (USP33/NF28) guidelines and it fulfilled the validation criteria. The method is sensitive, specific, fast, accurate, and requires minimum sample manipulation. It was applied on commercial A.S.K powder, to which all the active ingredients were separated from their excipients.

Keywords: Amprolium hydrochloride; Sulfaquinoxaline sodium; Vitamin K₃; ZIC-HILIC; Validation

Introduction

A.S.K Powder is a generic veterinary drug manufactured by Pharmacare pharmaceutical company (Palestine). The A.S.K powder is mainly used for the treatment and control of coccidiosis disease. It is very similar in composition to Amprocoxin-silv that is manufactured by Silvavet Company for veterinary medicines.

The A.S.K powder comprises Amprolium hydrochloride, Sulfaquinoxaline sodium and Vitamin K₃ sodium bisulfite (as menadione sodium bisulfite).

As shown in Figure 1, the three active ingredients of the A.S.K Powder are very hydrophilic and possess polar basic or acidic character. Therefore, under most circumstances, they would elute within the column void volume, thus resulting in a significant challenge to keep them retained when using reversed phase chromatography.

In order to enhance their retention without using derivatization methodologies or adding ion-pair reagent to the mobile phase, a new HPLC method which is based on zwitterionic hydrophilic interaction liquid chromatography (ZIC-HILIC) coupled with ultraviolet detection has been optimized and validated.

To our knowledge, there is no quality control method neither in the official Pharmacopoeias nor in the literature, that determines the drug active ingredients simultaneously and therefore A.S.K Powder was not registered at the Ministry of Health of Palestine till now.

There are different non-chromatographic and chromatographic methods, particularly RP-HPLC and ion-pair RP-HPLC, which report about the assay of each active ingredient individually or when present in combinations with other ingredients [1-17]. Some of these

Figure 1: The chemical structures for the three active ingredients in A.S.K Powder.

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methods suffer from drawback of using ion-pair reagents which include sensitivity reduction, extended time to reach equilibration, cleaning and regeneration of the column.

Therefore, there is a need to develop a fast, specific, and accurate method that allows the simultaneous determination of the three active ingredients within a reasonable retention time as per ICH/USP validation norms [18,19] (Figure 1).

Experimental Details

Chemicals

Samples of amprolium hydrochloride, sulfaquinoxaline sodium, vitamin K₁, sodium bisulfite reference standards were purchased from Sigma-Aldrich (Germany). Ammonium acetate extra pure, acetic acid (glacial), acetonitrile (ACN) and methanol HPLC grade solvents, hexane-1-sulfonic acid sodium salt and decane-1-sulfonic acid sodium salt were purchased from Merck (Germany).

High purified water was prepared by using a Millipore Milli-Q plus water purification system.

A.S.K Powder samples, and all the active ingredients and excipients usually used in manufacturing the pharmaceutical combination, were kindly supplied by Pharmarec pharmaceutical company, Palestine.

Equipments

Elite Lachrom high performance liquid chromatograph equipped with uv-detector and supported with autosampler and column oven and Elite Lachrom data system of Agilent (Merck Hitachi, England).

The Ultraviolet/visible spectrometer (PG Instruments, United Kingdom).

Chromatographic conditions

The chromatographic column used was a ZIC-HILIC column (25.0 cm x 4.6 mm) with particle size of 5 μm (Merck, Germany) protected with a ZIC-HILIC guard column (20 mm x 2.1 mm, 5 μm).

0.2 M ammonium acetate solution was prepared by dissolving 3.08 g of NH₄AC in purified water and diluted up to 200 ml with the same solvent.

The optimum mobile phase selected for the assay was prepared by mixing 0.2 M NH₄AC and acetonitrile (ACN) (15:85; v/v), shaken well and left tell the temperature of the mobile phase reached to the room temperature. Then the pH was adjusted to 5.7 by glacial acetic acid. The mobile phase was filtered using 0.45 μm microporous filter and was degassed by sonication prior to use. The standard and sample solutions were also filtered using 0.45 μm membrane filter.

A wavelength of 263 nm was chosen since it was found the most appropriate for the determination of the three active ingredients simultaneously. The flow rate used was 0.5 ml/minute as recommended by the column manufacturer whereby minimum height equivalent of theoretical plates and maximum number of theoretical plates are expected to be generated.

The injection volume was 20 µl and the temperature of the autosampler was 15°C and that of the column was 25°C. Total run time was about 18 minutes.

Preparation of stock and standard solutions

Stock solution for menadione sodium bisulfite was prepared by dissolving menadione sodium bisulfite reference standard equivalent to 20 mg menadione (vitamin K₁) in 80.0 ml of 90% ACN and diluting up to 100.0 ml with the same solvent. 5 ml of this solution was diluted up to 50 ml with 90% ACN to yield a solution with a final concentration of 20.0 μg/ml.

Standard solutions for amprolium HCl, sulfaquinoxaline sodium and vitamin K₁ were prepared by dissolving 20.0 mg of amprolium hydrochloride reference standard, 20 mg sulfaquinoxaline sodium reference standard in 70.0 ml of 90% ACN, 10 ml of menadione sodium bisulfite stock solution was added, mixed well and then diluted to 100.0 ml with 90% ACN. 5 ml of this solution was diluted up to 50 ml with mobile phase. The solution was filtered using 0.45 μm membrane filter. The obtained final solution contains 20 μg/ml for amprolium hydrochloride, 20 μg/ml sulfaquinoxaline sodium and 0.20 μg/ml vitamin K₁. This solution has been used within 24 hours and kept at 15°C and was protected from light.

Preparation of sample solution

Sample solution was prepared by dissolving 100 mg of A.S.K Powder in 80 ml of 90% ACN and then diluted up to 100 ml with the same solvent. Then 5 ml of the solution was diluted up to 50 ml with mobile phase. The latter solution was filtered using 0.45 μm membrane filter. The obtained final solution contains 20 μg/ml for amprolium hydrochloride, 20 μg/ml sulfaquinoxaline sodium and 0.20 μg/ml vitamin K₁. This solution has been used within 24 hours and kept at 15°C and was protected from light.

Results and Discussion

Development and optimization of the ZIC-HILIC-HPLC method

Since the combined active ingredients are highly hydrophilic, at first we have tried the ion pair RP-chromatography mode using sodium 1-hexanesulfonate and decane-1-sulfonic acid sodium salts at different concentration levels. Poor peak profile shapes with small resolution (Rs) values were obtained. Moreover, the ion pair reagents decreased the assay sensitivity for all ingredients particularly vitamin K₁ since it is present at 0.2% level of the overall mixture concentration. This unsatisfactory start, lead us to switch to ZIC-HILIC column which is designed to overcome the shortcomings of the ion-pair mode when separating very polar compounds in reversed phase chromatography.

The overlaid ultraviolet absorption spectra of the three active ingredients demonstrated that they shared a wavelength near to 263 nm, and therefore it was chosen during the entire study.

Different mobile phases have been employed in order to optimize the desired HPLC method using HILIC column. These mobile phases differ in the concentration of NH₄AC buffer, pH, and organic solvent strength.

The effect of NH₄AC buffer concentration: Our first choice of a mobile phase was ACN/10 mM NH₄AC buffer solution (80:20; v/v). The pH of the mobile phase was adjusted to 5.0. The ionic strength of the mobile phase started at 10 mM and was increased up to 200 mM (Figure 1). As the ionic strength of the mobile phase increased, the analysis time decreased and the resolution (Rs) increased among all separated drugs. When the concentration of NH₄AC was at 20 mM, peaks overlapped with no baseline separation between sulfaquinoxaline
sodium and vitamin K₃ ingredients. The amprolium hydrochloride eluted far away with a total analysis time of about 30 minutes, as shown in Figure 2.

At 75 mM, a new tiny peak was observed between sulfaquinoxaline sodium and vitamin K₃, which is due to a sulfaquinoxaline related impurity (Compound A) [4]. The best separation was achieved at mobile phase ionic strength of 200 mM. A resolution of >2.5 was achieved between sulfaquinoxaline sodium, its impurity and the other active ingredients with a total analysis time of 16 minutes. The achieved shorter retention time implies that the interaction is based on hydrophilic nature as the ionic strength increased. The positively charged ammonium ions in the mobile phase may weaken the hydrophilic interaction between the protonated ingredients and the stationary phase which result in shortening the retention time.

The effect of the mobile phase pH: A study of the pH effect on the resolution was deemed necessary to further optimize the separation conditions. The resolution values for all the ingredients under investigation were significantly influenced by the variation of the mobile phase pH. The pH range tested was from 3.5 up to 7.0. The best result was found at a pH between 5.6 and 5.8 (Figure 3). Maximum resolution for all ingredients was accomplished at pH 5.7.

As the pH increased, the retention time of all the ingredients slightly increased. The electrostatic interactions between the positively charged basic ingredients and the negatively charged surface silanol groups increased resulting in an increase of the retention time of all active ingredients. Final pH selected for the mobile phase was pH 5.7.

The effect of acetonitrile percentage: The effect of acetonitrile organic solvent percentages at fixed NH₄AC buffer concentration and mobile phase pH of 5.7 on retention and resolution was also investigated. It was found that increasing the ratio of ACN in the mobile phase resulted in an increase in the retention time and improved the peaks resolution. The best separation was achieved when 85% ACN was used (Figure 4).

By increasing the percentage of ACN, the polarity of the mobile phase decreased and the hydrophilic interactions between the analytes and the stationary were promoted, thereby dramatically increasing the retention of the ingredients.

The effect of acetonitrile on the retention time was systematically increased up to 90% ACN. However, when an extra 5% of ACN was added, the peak of vitamin K₃, eluted in a close proximity to amprolium HCl. This probably indicates a sudden disruption in the equilibrium coefficient of this compound in the HILIC column. As such ACN of 85% was selected for the mobile phase.

The effect of using methanol in addition to ACN as organic solvent modifier proved no significant effects on retention time and peaks resolution.

The effect of temperature

The effect of a column temperature on peak resolution and tailing factor was another chromatographic parameter that was studied during the optimization of HPLC methods. Different temperatures such as 15°C, 20°C, 25°C, 30°C were evaluated. It was found that varying temperatures between 15°C and 30°C had subtle influence on Rs values. The best tailing factor obtained was at temperatures of about 25°C and 30°C.

From all the accumulated results, the optimized mobile phase consists of a mixture of 0.2M NH₄AC solution and ACN organic solvent (15:85; v/v); at pH 5.7 at room temperature. During the method development process, the concentrations of the ingredients prepared were 20 µg/ml of sulfaquinoxaline sodium, 10 µg/ml of vitamin K₃ and 20 µg/ml of amprolium hydrochloride. Figure 5 shows typical chromatograms obtained for the combined standard mixture using the optimized conditions.
Method validation

The validation was carried out according to the ICH/USP guidelines [18,19]. Parameters such as specificity; linearity; range; accuracy (recovery); precision (repeatability and intermediate precision); robustness and stress test were all validated.

Specificity (placebo interference): The chromatograms of the placebo solution, standard and test solutions were recorded at the same wavelength in order to check the specificity of the method. No peaks were observed when the placebo, dextrose monohydrate, that lacks any chromophore was injected.

The retention time of the sulfaquinoxaline sodium, amprolium hydrochloride, and menadione sample peaks match exactly the peaks of the standard solution. No peaks were present at these retention times in the placebo chromatogram. Therefore, this method is suitable for the identification and quantification of the active ingredients in the A.S.K powder.

Linearity and range: Different amounts of sulfaquinoxaline sodium, amprolium hydrochloride and menadione (as sodium bisulfite) in the range of 60% to 130% of the labeled amount (5 concentration levels/3 replicates each) were added to A.S.K matrix (dextrose monohydrate).

The linearity for sulfaquinoxaline sodium, amprolium hydrochloride and vitamin K₃ within the range of 0.12 µg/ml to 0.26 µg/ml was investigated. The regression lines demonstrated linearity in the tested range. The regression lines were linear with (R²) of 0.9983 (Table 1).

As to vitamin K₃, the linearity was tested in a much smaller concentration within the range of 0.12 µg/ml to 0.26 µg/ml. The regression line was linear with (r) of 0.9998 for amprolium hydrochloride and vitamin K₃ showed results between 97.8% and 102% with RSD of less than 2.0% and therefore the acceptance criteria were fulfilled.

Precision

Repeatability: One laboratory analyst carried out the assay of sulfaquinoxaline sodium, amprolium hydrochloride and vitamin K₃ on six determinations of homogeneous sample of A.S.K powder at 100% level of the test concentration with the same analytical equipment at the same day. The repeatability results of the peak areas and statistical evaluation for assay of the three active ingredients is listed in table 3.

Intermediate precision (ruggedness): Two laboratory analysts carried out the assay of sulfaquinoxaline sodium, amprolium hydrochloride and vitamin K₃ on twelve homogeneous samples of A.S.K powder at 100% level of the final test concentration with different analytical equipments at different days. The assay results and statistical evaluation for assay of the three active ingredients is listed in table 3.

The RSD percentage values showed that the results of the assay of the three active ingredients are within a suitable intermediate precision for the specified range.

Robustness: Robustness of the investigated HILIC-HPLC new method included five deliberate variations to some chromatographic parameters as summarized in Table 4. The modifications include different mobile phase flow rates of 0.45, 0.50, and 0.55 ml/min and three different column temperatures in the range 23-27°C. Ammonium acetate buffer in the range of ±3 of the nominal value) were also investigated. Three column batches filled with the prescribed stationary phases were studied. Final three different pH values of the mobile

Table 1: Linear ranges of the three active ingredients: amprolium HCl, sulfaquinoxaline sodium and vitamin K₃ (menadione sodium bisulfite).

<table>
<thead>
<tr>
<th>Active ingredient</th>
<th>Regression equation</th>
<th>Correlation coefficient (R²)</th>
<th>Linear range (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amprolium HCl</td>
<td>y = 269332x + 62202</td>
<td>0.9998</td>
<td>(12-26)</td>
</tr>
<tr>
<td>Sulfaquinoxaline sodium</td>
<td>y = 597297x - 11291</td>
<td>0.9997</td>
<td>(12-26)</td>
</tr>
<tr>
<td>Vitamin K₃</td>
<td>y = 555682x - 644.34</td>
<td>0.9983</td>
<td>(0.12-0.26)</td>
</tr>
</tbody>
</table>

Table 2: Average recoveries, RSD values at five concentration levels of spiking (n=3).

<table>
<thead>
<tr>
<th>Active ingredient</th>
<th>Amount added (level %)</th>
<th>Average recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfaquinoxaline sodium</td>
<td>12µg/ml (60%)</td>
<td>99.2</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>16 µg/ml (80%)</td>
<td>99.7</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>20 µg/ml (100%)</td>
<td>98.3</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>24 µg/ml (120%)</td>
<td>99.2</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>26 µg/ml (130%)</td>
<td>99.5</td>
<td>0.06</td>
</tr>
<tr>
<td>Amprolium HCl</td>
<td>12 µg/ml (60%)</td>
<td>98.9</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>16 µg/ml (80%)</td>
<td>98.7</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>20 µg/ml (100%)</td>
<td>99.7</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>24 µg/ml (120%)</td>
<td>98.2</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>26 µg/ml (130%)</td>
<td>98.1</td>
<td>0.06</td>
</tr>
<tr>
<td>Vitamin K₃</td>
<td>0.12 µg/ml (60%)</td>
<td>102.0</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>0.16 µg/ml (80%)</td>
<td>99.5</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>0.20 µg/ml (100%)</td>
<td>97.8</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>0.24 µg/ml (120%)</td>
<td>100.5</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>0.26 µg/ml (130%)</td>
<td>101.5</td>
<td>0.32</td>
</tr>
</tbody>
</table>
Repeatability and the intermediate precision of the three active ingredients, as determined by HPLC articles to share and explore.

### Table 3: Repeatability and the intermediate precision of the three active ingredients.

<table>
<thead>
<tr>
<th>Active ingredient</th>
<th>Repeatability RSD% (n=6)</th>
<th>Intermediate Precision RSD% (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfaquinoxaline Sodium</td>
<td>0.77</td>
<td>0.66</td>
</tr>
<tr>
<td>Amprolium HCl</td>
<td>0.65</td>
<td>0.59</td>
</tr>
<tr>
<td>Vitamin K&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.82</td>
<td>0.90</td>
</tr>
</tbody>
</table>

Table 4: Robustness testing of the three active ingredients.

<table>
<thead>
<tr>
<th>Active ingredient</th>
<th>parameter</th>
<th>Flow rate (ml/min)</th>
<th>Temperature (°C)</th>
<th>[NH&lt;sub&gt;4&lt;/sub&gt;AC]</th>
<th>Column batches (Lots)</th>
<th>Mobile phase pH</th>
<th>Average assay (%) (n=3)</th>
<th>RSD% (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfaquinoxaline Sodium</td>
<td>Flow rate (ml/min)</td>
<td>100.9</td>
<td>100.8</td>
<td>101.6</td>
<td>101.3</td>
<td>0.67</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Temperature (°C)</td>
<td>100.5</td>
<td>100.8</td>
<td>101.0</td>
<td>101.0</td>
<td>0.49</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[NH&lt;sub&gt;4&lt;/sub&gt;AC]</td>
<td>1.01</td>
<td>1.26</td>
<td>0.12</td>
<td>0.67</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Column batches (Lots)</td>
<td>101.6</td>
<td>101.4</td>
<td>102.0</td>
<td>0.67</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mobile phase pH</td>
<td>101.3</td>
<td>101.0</td>
<td>100.9</td>
<td>0.47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amprolium HCl</td>
<td>Flow rate (ml/min)</td>
<td>100.0</td>
<td>100.0</td>
<td>101.8</td>
<td>102.0</td>
<td>0.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Temperature (°C)</td>
<td>100.0</td>
<td>100.6</td>
<td>0.65</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[NH&lt;sub&gt;4&lt;/sub&gt;AC]</td>
<td>1.01</td>
<td>0.69</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Column batches (Lots)</td>
<td>101.8</td>
<td>0.90</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Mobile phase pH</td>
<td>100.9</td>
<td>0.65</td>
<td></td>
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</table>

In summary, the proposed method can be used for the drug analysis in routine quality control of A.S.K formulation.

### Acknowledgments

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### References


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