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# Validation of a Stability-Indicating Assay of Amprolium Hydrochloride in Water Soluble Powder Formulation using Hydrophilic Interaction Liquid Chromatography

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**ABSTRACT**

A simple stability-indicating method was developed and validated to determine amprolium hydrochloride (HCl) in water soluble powder formulation. The method is based on zwitterionic hydrophilic interaction liquid chromatography (ZIC-HILIC) coupled with photo diode array detector (PDA). The separation was achieved on ZIC-HILIC column (250 mm × 4.6 mm, 5 µm) at 25°C temperature. The optimized mobile phase consisted of an isocratic solvent mixture of 200mM Ammonium acetate ($NH_4AC$) solution and acetonitrile (ACN) (25:75; v/v) with pH adjusted to 5.7 by glacial acetic acid. The mobile phase flow was fixed at 0.5 ml/min and the analytes were monitored at 267 nm. The effects of the chromatographic conditions on the peak retention, peak USP tailing factor and column efficiency were systematically optimized. Forced degradation experiments were carried out by exposing amprolium HCl standard and the water soluble powder formulation for thermal, photolytic, oxidative and acid-base hydrolytic stress conditions. The degradation products were well-resolved from the main peak and the excipients thus proving that the method is a reliable and stability-indicating. The method was validated as per ICH and USP guidelines and found to be adequate for the routine quantitative determination of amprolium hydrochloride in commercially available water soluble powder dosage forms.

## INTRODUCTION

Amprolium hydrochloride (HCl) water soluble powder is a veterinary drug which comprises amprolium hydrochloride as the active ingredient and dextrose monohydrate as an inactive excipient (Figure 1). It is used to control and treat coccidiosis in calves, sheep, goats, puppies, and birds. It is administered orally, often mixed with food (Papich, 2007).

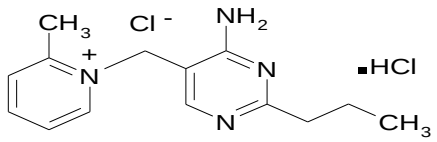


**Fig. 1.** Structure of Amprolium hydrochloride

Up to the present time, there is no stability indicating method to determine amprolium HCl either in bulk or in pharmaceutical dosage forms. The current adopted official British Pharmacopeial assay method is based on non-aqueous titration with perchloric acid which is not a stability-indicating method (British Pharmacopeia, 2013). The United States Pharmacopeial assay method is primarily based on utilizing high concentration of ion-pair reagent phase (12 g sodium 1-heptanesulfonate per liter of water) in the mobile phase to hold amprolium HCl from eluting with the dead volume of the column (USP, 2011).

Moreover, large amount of amprolium HCl standard was used (500µg/ml) since sensitivity was reduced significantly upon using the ion-pair reagent in the mobile phase. The large amount of ion pair reagent is not recommended since it shortens the life time of the column besides being very expensive.

Therefore, there is a need to develop and validate a stability-indicating quality control method that is sensitive, cheap and allows the determination of amprolium HCl in pharmaceutical formulations. Since amprolium HCl is a very polar compound and may elute with the dead volume using typical reversed phase column, zwitterionic hydrophilic interaction liquid chromatography (ZIC-HILIC) was utilized to directly determine amprolium HCl without using any ion-pair reagent additives.

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The developed HPLC method described herein successfully separates amprolium HCl from its all degradation products and from the placebo simultaneously. The method was validated according to the ICH/USP guidelines (ICH, 2005; USP, 2011).

## MATERIALS AND METHODS

Amprolium HCl reference standard 99.4% (Lot no: SZBA068X) was purchased from Sigma-Aldrich (Germany). Ammonium acetate extra pure, glacial acetic acid, HPLC grade acetonitrile and methanol solvents, hydrochloric acid fuming (37%), sodium hydroxide pellets and hydrogen peroxide (30%), were purchased from Merck (Germany). High purified water was prepared by using a Millipore Milli-Q plus water purification system. Ampro-bal 24% soluble powder samples (each one g contains 240 mg amprolium HCl) were purchased from local pharmacy, Palestine. Amprolium HCl active ingredient, dextrose monohydrate excipient was kindly supplied by Pharmacare pharmaceutical company, Palestine. ODS column (250 mm ×4.6 mm i.d., 5 µm particle) purchased from ACE, United Kingdom.

The HPLC system consisted of LaChrom (Merck-Hitachi) equipped with model L-7100 pump, L-7200 autosampler, L-7300 column oven, DAD L-7450 photo diode array (PDA) detector and D-7000 software HSM version 3.1 (Merck Hitachi, England). A double beam ultraviolet-visible spectrometer (PG Instruments, United Kingdom) was used. UV-Chamber (Model CM-10) Spectoline fluorescence analysis cabinet was used at 254nm.

The HPLC experimental conditions were optimized on a ZIC®-HILIC column (250 mm ×4.6 mm, 5µm) protected with a ZIC®-HILIC guard column (20mm× 2.1mm, 5µm) that was purchased from Merck, Germany. 200mM ammonium acetate solution was prepared by dissolving 3.08 g of $NH_4AC$ in high purified water and diluted up to 200 ml with the same solvent.

The optimum mobile phase was prepared by mixing 200 mM $NH_4AC$ solution and acetonitrile (ACN) (25:75; v/v), shaken well and left till the mobile phase reached to the room temperature. Then the pH was adjusted to 5.7 with glacial acetic acid. The mobile phase was filtered by using 0.45 µm microporous filter and was degassed by sonication prior to use. A wavelength of 267 nm was chosen.

The flow rate used was 0.5 ml/minute as recommended by the column manufacturer. The injection volume was 20 µl and the temperature of the column was 25° C. Total run time was about 11 minutes.

### Preparation of standard solution

The standard solution was prepared by dissolving 24 mg of amprolium hydrochloride reference standard in 80 ml of 80% ACN and diluting up to 100ml with the same solvent. Five ml of this solution was diluted up to 50 ml with mobile phase. This solution was filtered using 0.45 µm membrane filter before analysis. The obtained final solution contained 24µg/ml amprolium HCl. This solution was directly protected from light.

### Preparation of sample solution

The sample solution was prepared by transferring 100 mg of amprolium HCl water soluble powder into 100 ml volumetric flask. Then 80 ml of 80% ACN was added and shaken by mechanical means for 5 minutes, sonicated for two minute and then diluted up to 100 ml with the same solvent. Using volumetric pipette, 5 ml of this solution was transferred to 50 ml volumetric flask and completed to the volume using the mobile phase. This solution was filtered using 0.45 µm membrane filter before analysis. The obtained final solution contained 24 µg/ml amprolium HCl. This solution was directly protected from light.

### Forced degradation study

ICH prescribed stress conditions such as acidic, basic, oxidative, thermal and photolytic stresses were carried out.

#### *Standard drug stock solutions*

Forced degradation study was conducted on solutions that were prepared by transferring 24 mg amprolium HCl reference standard into four different 100 ml volumetric flasks. Then 80 ml of 80% ACN was added in each flask, shaken by mechanical means for 5 minutes and then sonicated for two minutes until completely dissolved. These stock solutions were kept at room temperature protected from light and used for forced degradation studies while the thermal degradation solution was prepared separately as shown below.

#### *Acid hydrolysis*

Ten ml of 1.0 N HCl was added into one of the flasks containing amprolium HCl stock solution, diluted to 100 ml with 80% ACN and kept at room temperature for 60 minutes in a dark place. Five ml of this solution was transferred into 50 ml volumetric flask, neutralized with 0.1 N NaOH and completed to volume using the mobile phase. This solution was filtered using 0.45 µm membrane filter before analysis. The obtained final solution contained 24 µg/ml amprolium HCl.

#### *Base hydrolysis*

Ten ml of 1.0 N NaOH was added into one of the flasks containing amprolium HCl stock solution, diluted to 100 ml with 80% ACN and kept at room temperature for 60 minutes in a dark place. Five ml of this solution was transferred into 50 ml volumetric flask, neutralized with 0.1 N HCl and completed to volume using the mobile phase. This solution was filtered using 0.45 µm membrane filter before analysis. The obtained final solution contained 24 µg/ml amprolium HCl.

#### *Oxidative hydrolysis*

Ten ml of 30% $H_2O_2$ was added into one of the flasks containing amprolium HCl stock solution, diluted to 100 ml with 80% ACN and kept at room temperature for 24 hours in a dark place. Five ml of this solution was transferred into 50 ml volumetric flask and completed to volume using the mobile phase.

This solution was filtered using 0.45 µm membrane filter before analysis. The obtained final solution contained 24 µg/ml amprolium HCl.

***Photo degradation***

One of the flasks containing amprolium HCl stock solution was studied separately for its photo degradation by exposing it to UV light at 254 nm in UV-Chamber for 36 hours and then diluted to 100 ml with 80% ACN. Five ml of this solution was transferred into 50 ml volumetric flask and complete to volume using the mobile phase. This solution was filtered using 0.45 µm membrane filter before analysis. The obtained final solution contained 24 µg/ml amprolium HCl.

***Thermal degradation***

The standard solution was prepared by transferring 24 mg amprolium HCl reference standard that have been previously kept at 105˚C in an oven for 72 hours, into 100 ml volumetric flasks. Then 80 ml of 80% ACN was added to the flask, shaken by mechanical means for 5 minutes and sonicated for two minutes until completely dissolved. Five ml of this solution was diluted up to 50 ml with mobile phase. This solution was filtered using 0.45 µm membrane filter before analysis. The obtained final solution contained 24µg/ml amprolium HCl.

**Forced degradation study on amprolium HCl water soluble powder**

The sample stock solutions were prepared by separately transferring 100 mg of the amprolium HCl water soluble powder (containing 240 mg amprolium HCl per g) into series of five different 100 ml volumetric flasks. The very same procedure adopted for the standard solutions was used in the amprolium HCl water soluble powder. The obtained final solution contained 24 µg/ml amprolium HCl.

## RESULT AND DISCUSSION

***Method development and Optimization***

Prior using the ZIC-HILIC column, different acetonitrile (ACN) concentrations at fixed ammonium acetate ($NH_4AC$) buffer ionic strength of 0.2M adjusted to pH 5.5 was tried on a typical C18-reversed phase column. Amprolium HCl peak always eluted near to the dead volume even when the concentration of ACN reduced to 3.0%. To enhance the retention of amprolium HCl, sodium 1-heptanesulfonate ion pair reagent was tried on the same C18 column. The mobile phase was a mixture of 1.0 g of sodium 1-heptanesulfonate dissolved in 500 ml of water; 12 ml of glacial acetic acid, 2.0 ml of triethylamine, with different percentages of methanol ranging from 50 to 450 ml using 100 ml increments. A broad amprolium HCl peak with a tailing factor of 3.4 was produced at capacity factor (k') of less than 1.5. The sensitivity decreased significantly to less than 70% when compared to a free ion-pair mobile phase. The developed ZIC-HILIC-HPLC method was tested for the effect of ACN strength, pH, temperature and $NH_4AC$ concentration. Ionic strength of 50mM $NH_4AC$ concentration was increased up to 200mM with 50mM increment at fixed pH of 5.5. It was noticed that increasing the concentration of $NH_4AC$ improved the tailing factor of amprolium HCl and decreased the retention time. Different pH values from 3.7 up to 7.2 with 0.5 increments were also evaluated. As the pH increased, the retention time of amprolium HCl slightly increased. The best tailing factor with reasonable retention time was obtained at pH of 5.7. The effect of acetonitrile strength on retention and tailing factor of amprolium HCl was investigated using 200mM $NH_4AC$ at pH of 5.7. As the ACN percent increased from 55% to 95% with 10% increment, the retention time increased**.** The best peak tailing factor with reasonable retention time of about 11 minutes was obtained at 75% ACN. Different temperatures of 15°C, 20°C, 25°C, 30°C, and 35°C were evaluated. Results indicate that temperature at this range does not play a substantial role in the retention or the peak shape of amprolium HCl and therefore a temperature of 25°C was chosen during the entire study.

The optimal mobile phase chosen with ZIC-HILIC column was an isocratic solvent mixture prepared by mixing 200 mM $NH_4AC$ solution and ACN (25:75; v/v), shaken well and left till the mobile phase reached to the room temperature. Then the pH was adjusted to 5.7 with glacial acetic acid. A wavelength of 267 nm was chosen since amprolium HCl was found to have a maximum at this wavelength. Figure 2 and 3 show typical chromatogram of the placebo and freshly prepared standard of amprolium HCl used at the optimized conditions respectively.

**Method Validation**

After the successful optimization of the ZIC-HILIC-HPLC method, it was validated in accordance to the ICH/USP guidelines (ICH, 2005, USP, 2011). Parameters such as system suitability, specificity (placebo and forced degradation interferences), sensitivity (LOD and LOQ), linearity, range, accuracy (recovery), precision (repeatability and intermediate precision), robustness and stability indicating capability were validated.

**System suitability**

The system suitability was determined by injecting successive six replicates of the same standard solution and analyzing the amprolium HCl for its peak area, peak USP tailing factor, number of theoretical plates and capacity factor. The system suitability results for a solution of 24 µg/ml amprolium HCl revealed %RSD of 0.81% for peak areas. This method meets the accepted requirements as shown in table 1.

**Table. 1:** Summary of the accepted system suitability requirements .

| Parameter | Amprolium HCl | Accepted limit* |
|---|---|---|
| % RSD | 0.81 | ≤ 2.0% |
| Tailing factor ($T_f$) | 1.32 | ≤ 2.0 |
| Number of theoretical plates (N) | 9118 | ≥3000 |
| Capacity factor (k') | 3.5 | ≥2.0 |

*Set according to Palestinian Ministry of Health Registration Department criteria

**Table. 2:** Summary of the forced degradation of amprolium HCl standard and amprolium HCl water soluble powder.

| Name | Stress condition | Degradation% | Purity index* |
|---|---|---|---|
| **Amprolium HCl standard** | Acidic/0.10 N HCl / 60 min at RT | 5.87 | 1.0000 |
| | Alkaline/0.10 N NaOH / 60 min at RT | 91.43 | 0.9987 |
| | Oxidative/3.0% $H_2O_2$ /24 hours at RT | 28.74 | 0.9993 |
| | Thermal/105 ℃/72 hours | 3.56 | 1.0000 |
| | Light/ UV-254nm /36 hours | 4.63 | 1.0000 |
| **Amprolium HCl sample** | Acidic/0.10 N HCl / 60 min at RT | 5.73 | 0.9999 |
| | Alkaline/0.10 N NaOH / 60 min at RT | 91.92 | 0.9989 |
| | Oxidative/3.0 % $H_2O_2$ /24 hours at RT | 28.23 | 0.9991 |
| | Thermal/105 ℃/72 hours | 3.51 | 0.9999 |
| | Light/ UV-254nm /36 hours | 4.69 | 1.0000 |

* The accepted value is > 0.990 that set according to Palestinian Ministry of Health Registration Department criteria. The purity index is a measure of spectral heterogeneity of a peak.

**Table. 3:** Regression statistics.

| Active ingredient | Linearity range (µg/ml) | $R^2$ | Linearity equation* | Y-intercept (%) |
|---|---|---|---|---|
| **Amprolium HCl** | 12-36 | 0.9998 | Y=270667X+4614.8 | 0.07% |

***Y is the dependent variable and X is the independent variable***

**Table. 4:** Average recoveries at five concentration levels of spiking of amprolium HCl.

| Active ingredient | Amount added (level %) | Average recovery (%) ± S.D (n=3) |
|---|---|---|
| **Amprolium HCl** | 12 µg /ml (50%) | 98.6 ± 0.60 |
| | 18 µg /ml (75%) | 98.8 ± 0.65 |
| | 24 µg /ml (100%) | 99.6 ± 0.73 |
| | 30 µg /ml (125%) | 99.2 ± 0.85 |
| | 36 µg /ml (150%) | 100.3 ± 1.13 |

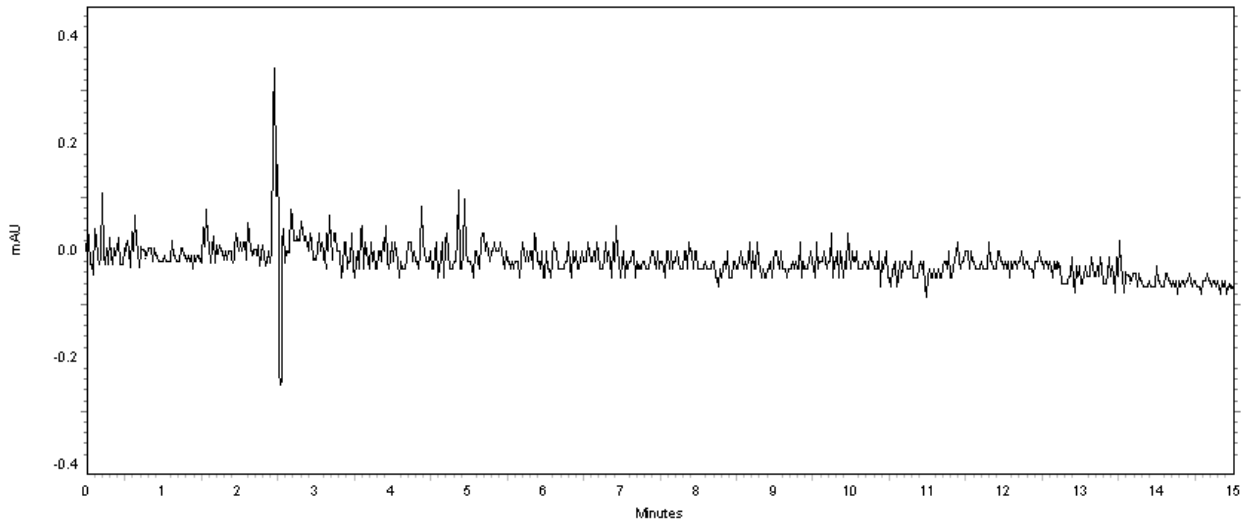


**Fig. 2:** Typical chromatogram of placebo (Dextrose monohydrate).

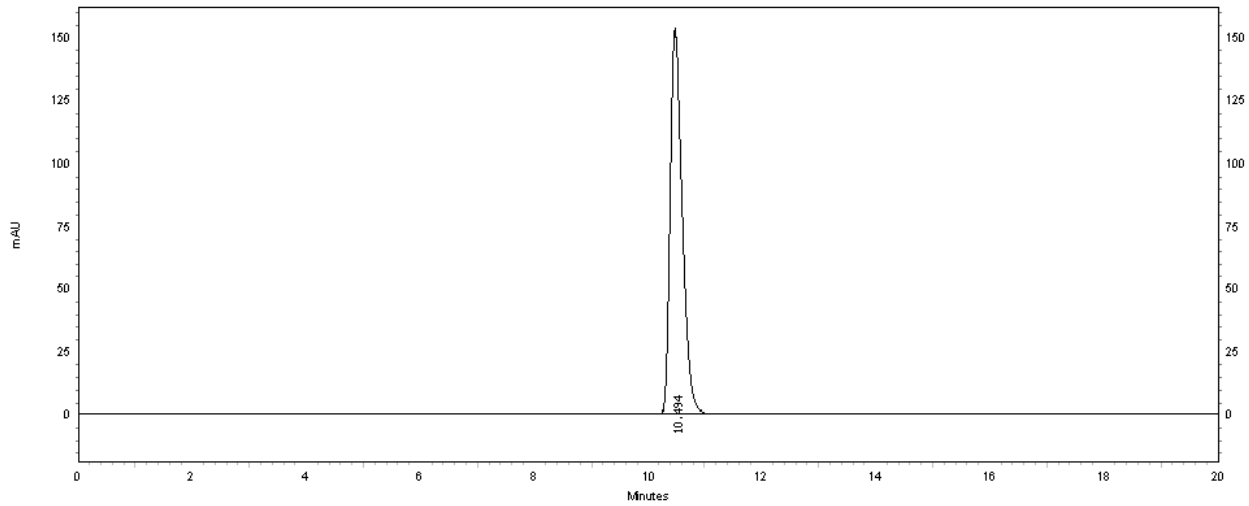


**Fig. 3:** Typical chromatogram of a standard of 24 µg/ml amprolium HCl.

**Specificity (placebo and forced degradation interference)**

Generally the specificity of a method is its suitability for the analysis of a compound in the presence of potential impurities. Placebo, standard and sample test solutions were all injected at the same wavelength of 267 nm to assure the specificity of the optimized method. A comparison of the retention times of amprolium HCl in sample solution and in the standard solution were exactly the same. Figures 2 and 3 showed that there is no interference at the retention time of amprolium HCl due to the placebo. Therefore, the proposed method is suitable for the quantification of the amprolium HCl in amprolium HCl water soluble powder. The specificity of the method to amprolium HCl was determined in the presence of its stress impurities. It was assessed by performing forced degradation studies on pure standards of the amprolium HCl separately to indicate the initial results and on samples of amprolium HCl water soluble powder in presence of its potential degradants. The stress conditions studied are UV-light (254 nm), heat (105°C), acid hydrolysis (0.10 N HCl), base hydrolysis (0.10 N NaOH) and oxidation (3% $H_2O_2$). The stressed sample solutions were analyzed against freshly prepared standard and sample solutions. The assay and purity check for the stressed standard and sample solutions were calculated as summarized in Table 2. Table 2 revealed that the alkaline and oxidative stress results showed extensive degradation in comparison to other stress conditions. Peak purity index for amprolium HCl was found to be not less than 0.9987, a higher value than the accepted limit (0.990). Therefore, there was no interference between the amprolium HCl peak and any other stress impurity peaks in the chromatogram. Almost the same pattern of degradation was obtained for amprolium HCl in the amprolium HCl water soluble powder samples. Figures (4-8) show the chromatographic profiles of the amprolium HCl and the degradation products after exposing the amprolium HCl water soluble powder to different stress conditions as in Table 2.

**Sensitivity**

The sensitivity of the method was explored via measuring the limit of detection (LOD) and the limit of quantitation (LOQ) for amprolium HCl at a signal-to-noise ratio of 3 and 10 respectively. It has been achieved by injecting a series of diluted solutions with known concentrations. LOD was found to be 0.015 µg/ml. LOQ was found to be 0.05µg/ml with RSD of 4.2% (accepted value is less than 10%).

**Linearity and range**

Different amounts of amprolium HCl in the range of 50% to 150% of the labeled amount (5 concentration levels and 3 replicates each) were spiked to amprolium HCl water soluble powder matrix (Placebo). The linearity in the range of 12-36 µg/ml for amprolium HCl was investigated. The regression line demonstrated linearity in the tested range. The regression analysis confirmed that the deviation of the y-intercept from zero is not significant; and the regression line was linear with $R^2$ of 0.9998 (Figure 9, Table 3).

**Accuracy (recovery)**

Accuracy was determined by the recovery study of known amounts of amprolium HCl standard added to a placebo matrix of water soluble powder dosage form. Different concentrations of the amprolium HCl were added to placebo matrix and the recovery was measured. The accuracy as reflected from recovery data of the amprolium HCl is listed in Table 4.

The average recovery data of amprolium HCl showed results between 98.6% and 100.3% which are within the acceptable limit of (98.0 to 102.0% as set according to Palestinian Ministry of Health Registration Department criteria).

***Repeatability***

One laboratory analyst carried out the assay of amprolium HCl on six determinations of homogeneous sample of amprolium HCl water soluble powder dosage form at 100% level of the test concentration with the same analytical equipment at the same day. The assay results and statistical evaluation for assay of the amprolium HCl revealed %RSD value of 0.65% which is within the acceptable limit of 2.0% (Set according to Palestinian Ministry of Health Registration Department criteria).

***Intermediate Precision (ruggedness)***

Two laboratory analysts carried out the assay of amprolium HCl on twelve homogeneous samples of amprolium HCl water soluble powder at 100% level of the final test concentration with two different analytical equipments on two different days. The assay results and statistical evaluation for assay of the amprolium HCl revealed % RSD value of 1.38% which is within the acceptable limit of 2.0%. The results of the assay of the amprolium HCl proved that the method is repeatable and rugged enough for day to day use.

**Robustness**

Premeditate variations were performed in the experimental conditions of the HPLC method to assess its robustness. The six variations imposed to the chromatographic method are summarized in Table 5. 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 22-28°C.

Different $NH_4AC$ solution concentrations in mobile phase (190mM, 200mM and 210mM) and different ACN percentages in mobile phase (73%, 75% and 77%) were also investigated. Two column batches filled with the same prescribed stationary phases were studied. Finally, three different pH values of the mobile phase at 5.5, 5.7, and 5.9 were tested. The assay results of amprolium HCl showed results between 98.6% and 101.5% which are within the acceptable limit of (98.0 to 102.0%) (Table 5).

**Table. 5:** Robustness testing of the amprolium HCl active ingredient.

| Active ingredient | Parameter | Modification | Average assay%±S.D *(n=3)* |
|---|---|---|---|
| **Amprolium HCl** | Flow rate (ml/min) | 0.45 | 100.7 ± 0.79 |
| | | 0.50 | 100.9 ± 0.93 |
| | | 0.55 | 99.5 ± 0.81 |
| | Mobile phase ratio (v/v) $NH_4AC$: ACN | 27:73 | 99.8 ± 1.02 |
| | | 25:75 | 100.3 ± 0.93 |
| | | 23:77 | 100.9 ± 0.89 |
| | Temperature ($^oC$) | 22°C | 99.9 ± 1.04 |
| | | 25°C | 101.2 ±0.80 |
| | | 28°C | 100.9 ±0.93 |
| | $NH_4AC$ Conc.(mM) | 190 | 99.2 ±0.63 |
| | | 200 | 99.6 ±0.71 |
| | | 210 | 98.6 ±0.58 |
| | Mobile phase pH | 5.5 | 100.4 ±0.97 |
| | | 5.7 | 101.1 ±0.67 |
| | | 5.9 | 101.5 ±0.48 |
| | Column batches | L010129977 | 100.3 ± 0.89 |
| | | L010134877 | 100.8 ± 0.73 |

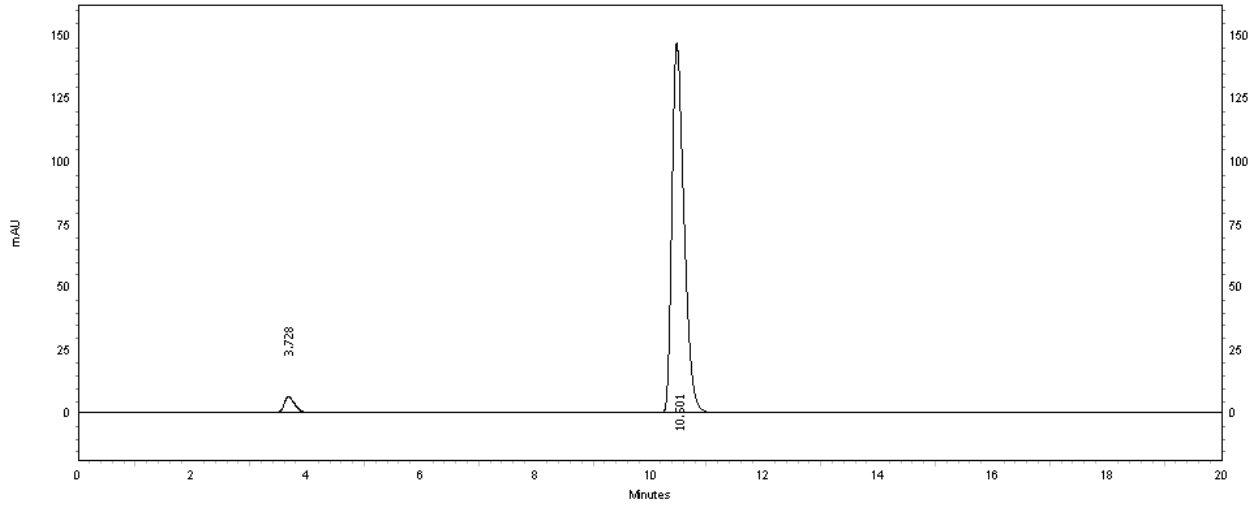


**Fig. 4:** Typical chromatogram of the amprolium HCl water soluble powder upon exposure to UV-light.

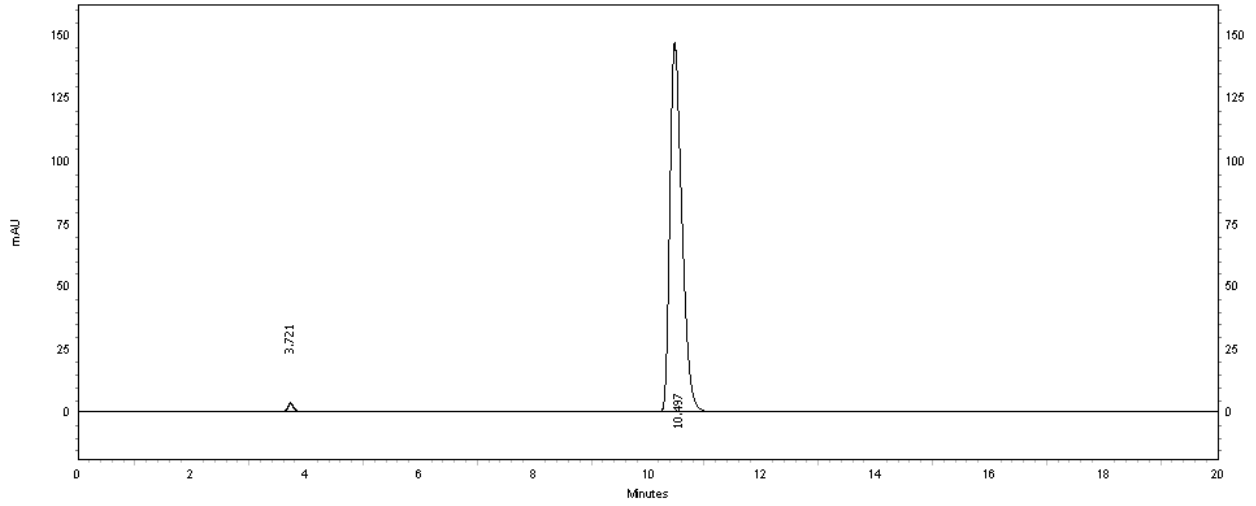


**Fig. 5:** Typical chromatogram of the amprolium HCl water soluble powder upon exposure to heat.

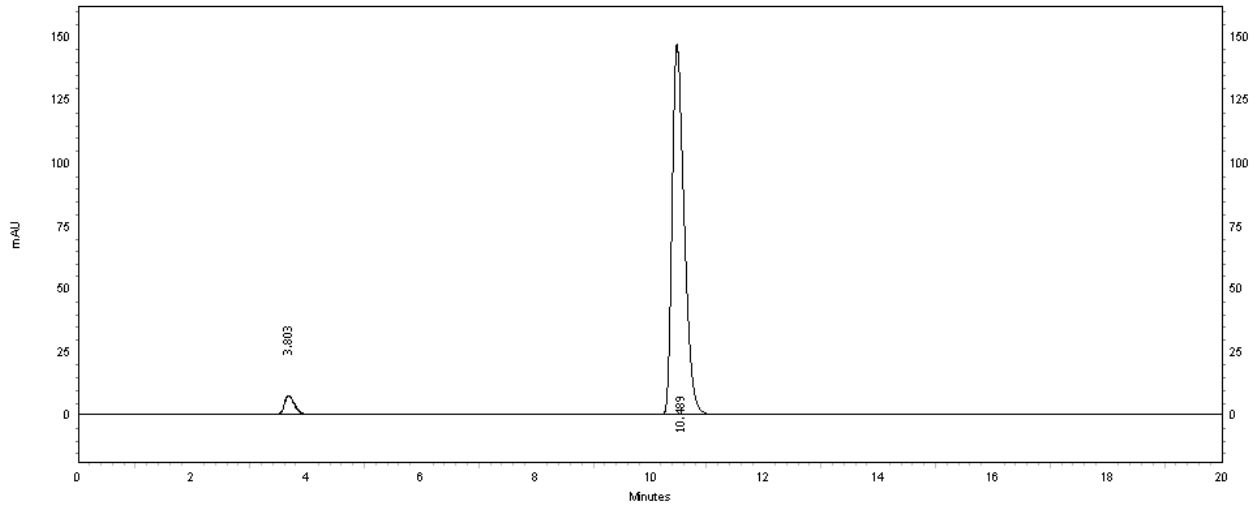


**Fig. 6:** Typical chromatogram of acidic degradation of the amprolium HCl water soluble powder.

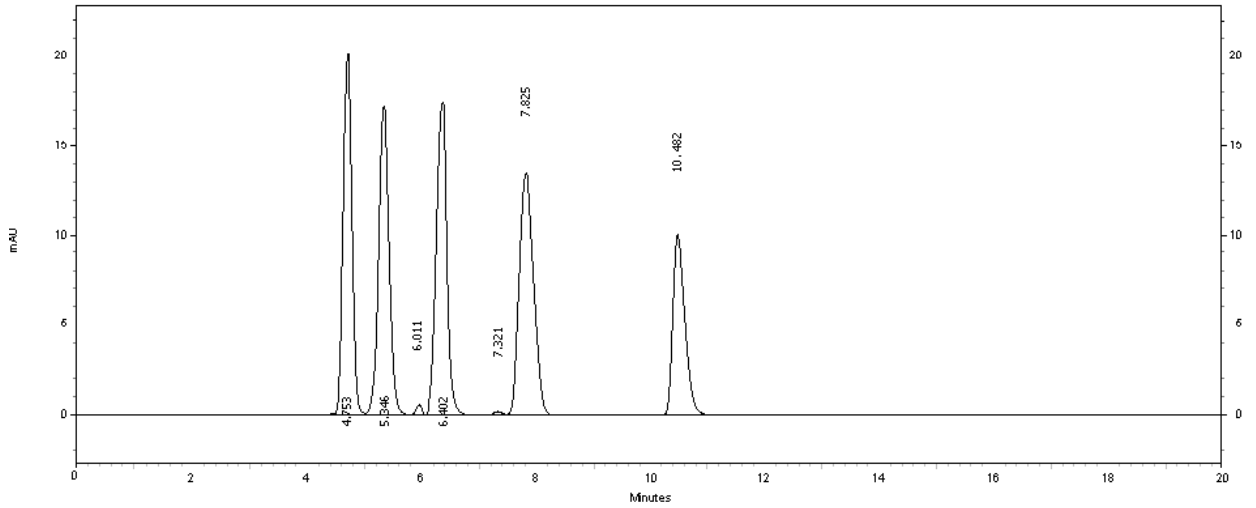


**Fig. 7:** Typical chromatogram of basic degradation of the amprolium HCl water soluble powder.

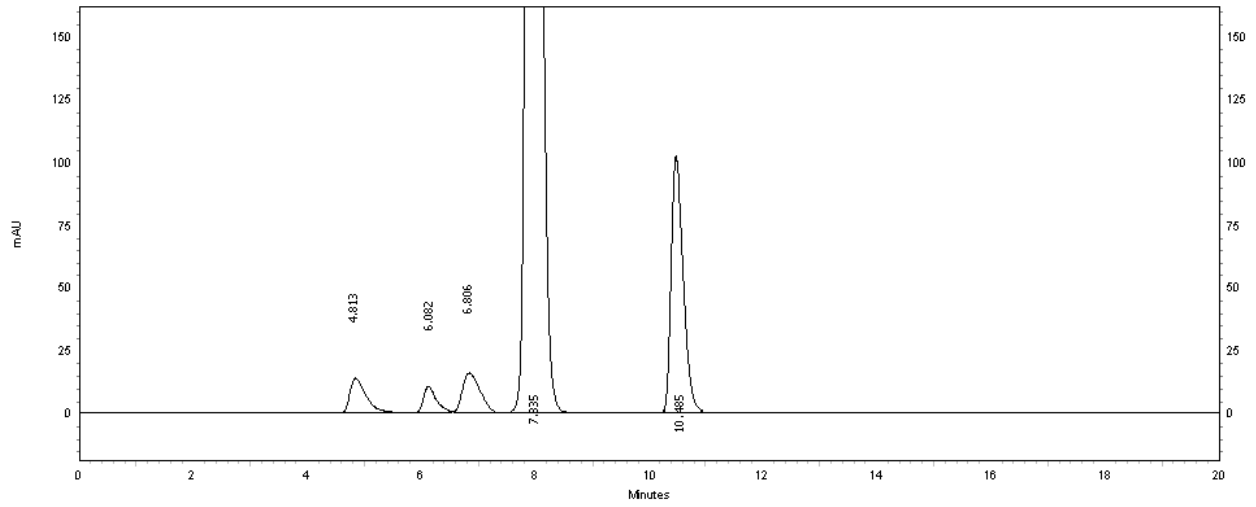


**Fig. 8:** Typical chromatogram of oxidative degradation of the amprolium HCl water soluble powder, the peak at 7.835 minutes is due to $H_2O_2$

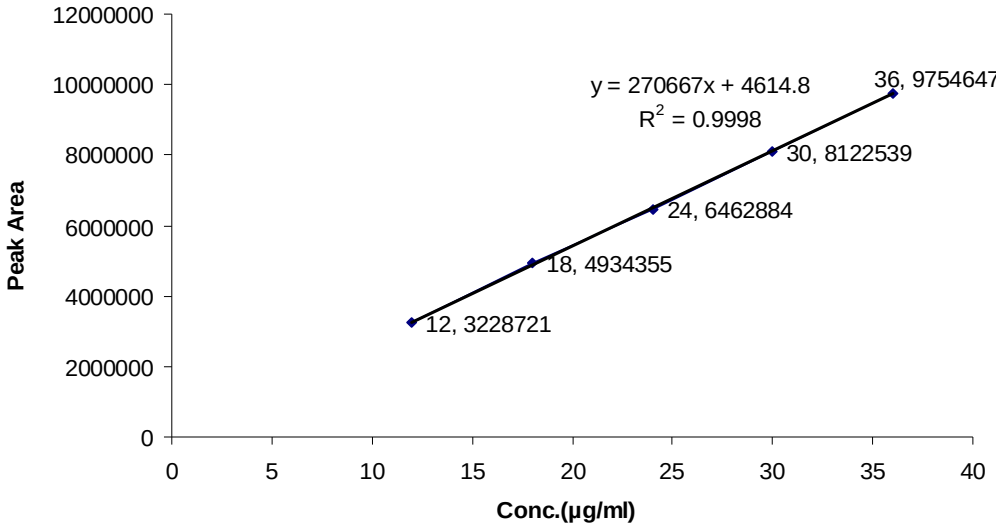


**Fig. 9:** Linearity and range for Amprolium HCl.

**Applicability of the method to a marketed product**

It is evident from the results obtained that the validated method gave satisfactory results with respect to the analysis of amprolium HCl. The validated method is applied to commercially available product (Ampro-bal 24% soluble powder) as shown in Table 6.

**Table. 6:** Result of a market product

| Product Name | Labeled claim (mg/g) | Amprolium HCl (mg/g) | Assay% |
|---|---|---|---|
| Ampro-bal 24% soluble powder | 240mg/g | 243.4 | 101.4% |

This acceptable value indicated the applicability of the proposed method for the routine quality control of amprolium HCl water soluble powder.

## CONCLUSION

The validated HPLC method developed for the quantitative quality control determination of amprolium HCl in its water soluble powder was evaluated over system suitability, specificity, sensitivity, linearity, range, accuracy (recovery), precision (repeatability and intermediate precision) and robustness. All the validation results were within the allowed specifications of ICH/USP guidelines. The developed method proved to be accurate, very sensitive and stability indicating for the determination of the amprolium HCl in its water soluble powder in the presence of excipients and the degradation products. The assay showed complete separation of amprolium HCl from its degradation products and from the placebo. As a result, the proposed ZIC-HILIC-HPLC method could be adopted for quantitative quality control and routine analysis of amprolium HCl water soluble powder.

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