

## DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF SULFAQUINOXALINE SODIUM IN WATER SOLUBLE POWDER FORMULATION

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### ABSTRACT

**Objective:** This study aims to develop and validate a stability-indicating hydrophilic interaction liquid chromatographic method for the determination of sulfaquinoxaline sodium (SQXS) in the presence of sulfaquinoxaline related compound A (SQXA) in a commercially available water soluble powder formulation.

**Methods:** The analytes were separated on zwitterionic hydrophilic interaction liquid chromatography (ZIC-HILIC) column with a solvent mixture of 200mM ammonium acetate (NH<sub>4</sub>AC) solution and acetonitrile (ACN) (10:90; v/v) at 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 263nm at ambient temperature. Forced degradation experiments were carried out by exposing sulfaquinoxaline sodium standard and the water soluble powder formulation for thermal, photolytic, oxidative and acid-base hydrolytic stress conditions.

**Results:** The results showed that SQXA and the other degradation products were fully resolved from sulfaquinoxaline sodium and thus the proposed ZIC-HILIC method is stability-indicating.

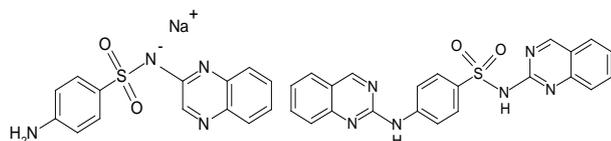
**Conclusion:** The method was validated as per ICH and USP guidelines and found to be adequate for routine quantitative determination of SQXS in the presence of SQXA and other degradation impurities in commercially available water soluble powder dosage forms.

**Keywords:** Validation, Stability-indicating method, Sulfaquinoxaline sodium, Sulfaquinoxaline related compound A, Water soluble powder.

### INTRODUCTION

Sulfaquinoxaline sodium water soluble powder is a veterinary drug which contains sulfaquinoxaline sodium (SQXS) active ingredient and dextrose monohydrate inactive excipient. It is an oral bacteriostatic antibiotic and coccidiostat. It is used for the prevention and treatment of intestinal coccidiosis in rabbits and hepatic coccidiosis due to *Eimeria Stiedae* [1].

The main associated impurity of SQXS is the sulfaquinoxaline related compound A (SQXA) as shown in Figure 1.



Sulfaquinoxaline sodium (SQXS) Sulfaquinoxaline related compound A (SQXA)

Fig. 1: Structures of SQXS and SQXA

Several sulfonamide residues including SQXS were screened and quantified in bovine, porcine, chicken tissues, whole egg, milk and veterinary drugs using HPLC or LC-MS methods [2-6]. A novel hydrophilic interaction liquid chromatographic (HILIC-HPLC) method for the simultaneous determination of SQXS combined with amprolium hydrochloride and vitamin K3 in powder formulation was validated [7]. More recently, we have developed and validated stability-indicating RP-HPLC method for the simultaneous determination of Sulfadiazine sodium (SDZS), Sulfathiazole sodium (STZS) and Sulfadimidine sodium (SDMS) in water soluble powder dosage form [8]. The current official British Pharmacopoeial (BP) assay of sulfaquinoxaline free base is based merely on titration with sodium nitrite reagent and therefore is not adequate as stability indicating [9]. The United States Pharmacopoeial (USP) assay however is based on the determination of sulfaquinoxaline free base using reversed phase chromatography and the related SQXA compound which is present in considerable quantity is only

determined by TLC [10]. Moreover, water, acetonitrile, glacial acetic acid, tetrahydrofuran, and ammonium hydroxide were utilized in the mobile phase with large concentration of SQXS standard (700µg/ml) to enhance its sensitivity [10].

Although, many reversed phase HPLC methods have been reported for the estimation of SQXS, but there is no stability indicating method to determine SQXS in the presence of SQXA and other degradation impurities either in bulk or in pharmaceutical dosage forms.

Therefore, there is a need to develop and validate a stability-indicating quality control method that is simple, sensitive and allows the quantitative determination of SQXS in pharmaceutical formulations in the presence of SQXA or any other degradation products.

The developed HILIC-HPLC method described herein successfully separates SQXS from SQXA, from all the degradation products and from the placebo simultaneously. Because analytical methods must be validated before use by the pharmaceutical industry, the proposed method was validated in accordance with ICH/USP guidelines [11-12].

### MATERIALS AND METHODS

#### Materials and Reagents

The reference standard of SQXS (99.9%) (Lot no: BCBB7740V) was purchased from Sigma-Aldrich (Germany) and the USP SQXA (96%) (Lot number: F0E093) was also used. Extra pure ammonium acetate, 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. Sulfaquinoxaline sodium soluble powder samples (each one gram contains 250 mg SQXS), SQXS active ingredient, and dextrose monohydrate excipient were kindly supplied by Pharmacare pharmaceutical company, Palestine. ODS column (250 mm ×4.6 mm i.d., 5 µm particle) purchased from ACE, United Kingdom. A ZIC®-HILIC column (250 mm ×4.6 mm, 5µm)

protected with a ZIC®-HILIC guard column (20mm× 2.1mm, 5µm) was purchased from Merck, Germany.

### Equipments

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.

### Chromatographic conditions

Ammonium acetate solution (200mM) was prepared by dissolving 3.08 g of NH<sub>4</sub>AC 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<sub>4</sub>AC solution and acetonitrile (ACN) (10:90; 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 263 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 ten minutes.

### Preparation of system suitability solution

The system suitability solution was prepared by transferring 25 mg of SQXS and 6 mg of SQXA reference standards into 100 ml volumetric flask. Then 80 ml of MeOH 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 25µg/ml SQXS and 6µg/ml SQXA. This solution was directly protected from light.

### Preparation of standard solution

The standard solution was prepared by transferring 25 mg of SQXS reference standard into 100 ml volumetric flask. Then 80 ml of MeOH 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 25µg/ml SQXS. This solution was directly protected from light.

### Preparation of sample solution

The sample solution was prepared by transferring 100 mg of sulfaquinoxaline sodium water soluble powder into 100 ml volumetric flask.

Then 80 ml of MeOH 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 25 µg/ml SQXS. 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 25 mg SQXS reference standard into four different 100 ml volumetric flasks.

Then 80 ml of MeOH 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 SQXS stock solution, diluted to 100 ml with MeOH 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 25 µg/ml SQXS.

### Base hydrolysis

Ten ml of 1.0 N NaOH was added into one of the flasks containing SQXS stock solution, diluted to 100 ml with MeOH 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 0.45µm membrane filter before analysis. The obtained final solution contained 25µg/ml SQXS.

### Oxidative hydrolysis

Ten ml of 30% H<sub>2</sub>O<sub>2</sub> was added into one of the flasks containing SQXS stock solution, diluted to 100 ml with MeOH 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 25 µg/ml SQXS.

### Photo degradation

One of the flasks containing SQXS 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 MeOH. 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 25 µg/ml SQXS.

### Thermal degradation

The standard solution was prepared by transferring 25 mg SQXS reference standard that has been previously kept at 105°C in an oven for 72 hours, into 100 ml volumetric flask. Then 80 ml of MeOH was added to the flask, shaken by mechanical means for 5 minutes and sonicated for two minutes until completely dissolved and then diluted up to 100 ml with the same solvent. 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 25µg/ml SQXS.

### Forced degradation study on sulfaquinoxaline sodium water soluble powder

The sample stock solutions were prepared by separately transferring 100 mg of the sulfaquinoxaline sodium water soluble powder (containing 250 mg SQXS per g) into series of five different 100 ml volumetric flasks.

The very same procedure adopted for the standard solutions was used in the sulfaquinoxaline sodium water soluble powder. The obtained final solution contained 25 µg/ml SQXS.

## RESULT AND DISCUSSION

### Method development and Optimization

The proper choice of a stationary phase greatly depends on the chemical structure of the target pharmaceutical drug. Reversed

phase ODS column was first used at different acetonitrile (ACN) concentrations at fixed ammonium acetate (NH<sub>4</sub>AC) buffer ionic strength of 0.2M adjusted to pH 5.5. The SQXS peak eluted near to the void and no separation was obtained between the SQXS and SQXA even when the concentration of ACN reduced to 3.0%. Therefore, zwitterionic hydrophilic interaction liquid chromatography (ZIC-HILIC) was utilized to directly separate SQXS and SQXA without using any ion-pair reagent additives.

The developed ZIC-HILIC-HPLC method was tested for the effect of ACN strength, pH, NH<sub>4</sub>AC concentration and temperature. Ionic strength of 50mM NH<sub>4</sub>AC concentration was increased up to 200mM with 50mM increment at fixed pH of 5.5. It was noticed that increasing the concentration of NH<sub>4</sub>AC improved the resolution between SQXS and SQXA through decreasing the retention time of SQXS and increasing the retention time of SQXA. Different pH values from 3.7 up to 7.2 with 0.5 increments were also tested. As the pH increased, the retention time of SQXS decreased and that of SQXA increased.

The best tailing factor and resolution were obtained at pH of 5.7. The effect of acetonitrile strength on resolution and tailing factor of SQXS was investigated using 200mM NH<sub>4</sub>AC at pH of 5.7. As the ACN percent increased from 55% to 95% with 5% increment, the retention times of both SQXS and SQXA increased and the resolution improved.

The best peak tailing factor and resolution with run time less than ten minutes were obtained at 90% 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 any substantial role in the retentions or the peaks shape of SQXS or SQXA 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<sub>4</sub>AC solution and ACN (10:90; 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 263 nm was chosen since SQXS and SQXA were found to have maxima at this wavelength. Figure 2 and 3 show typical chromatogram of the placebo and freshly prepared system suitability solution of SQXS and SQXA used at the optimized conditions, respectively.

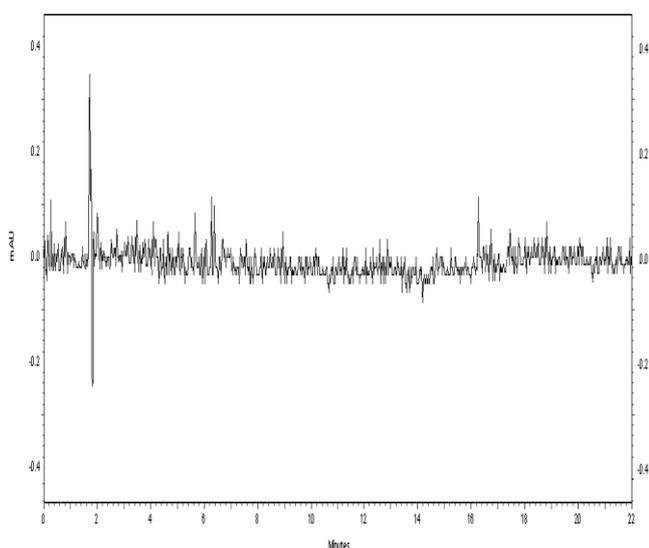


Fig. 2: Zoomed view of typical placebo chromatogram.

**Method Validation**

The optimized ZIC-HILIC-HPLC method was validated as per ICH/USP guidelines. 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.

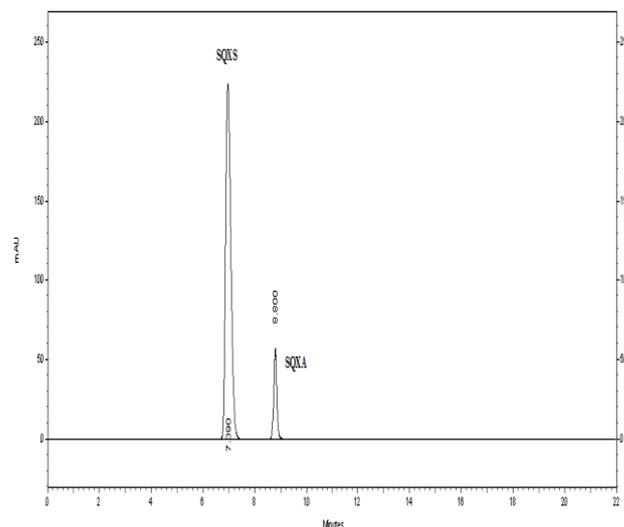


Fig. 3: Chromatogram of a standard mixture of SQXS (25 µg/ml) and SQXA (6µg/ml).

**System suitability**

The system suitability was determined by injecting successive six replicates of the same system suitability solution and analyzing the SQXS and SQXA for their peaks area, peaks USP tailing factor, resolution, number of theoretical plates and capacity factors. The system suitability results for a solution of 25 µg/ml SQXS and 6µg/ml of SQXA revealed %RSD of 0.83% and 1.31% for peaks areas, respectively. This method meets the accepted requirements as set by the Palestinian Ministry of Health Registration Department (table 1).

Table 1: Summary of the accepted system suitability requirements

Parameter	SQXS	SQXA	Accepted limit
% RSD	0.83	1.31	≤ 2.0%
Tailing factor (T <sub>f</sub> )	1.28	1.36	≤ 2.0
Resolution (R <sub>s</sub> )	---	3.1	≥ 2.0
Number of theoretical plates (N)	6235	26804	≥ 2000
Capacity factor (k')	2.9	3.9	≥ 2.0

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

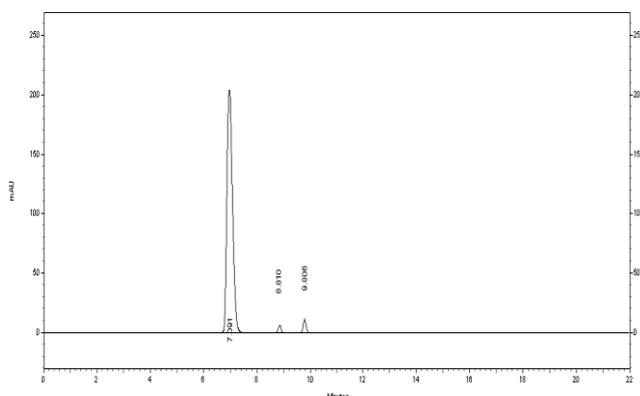
Generally the specificity of a method is its suitability for the analysis of a compound in the presence of potential impurities. Placebo, standard and sample test solutions were all injected at the same wavelength of 263 nm to assure the specificity of the optimized method. A comparison of the retention time of SQXS in sample solution and in the standard solution was exactly the same. Figures 2 and 3 showed that there is no interference at the retention time of SQXS due to the placebo. Therefore, the proposed method is suitable for the quantification of the SQXS in sulfaquinoxaline sodium water soluble powder. The specificity of the method to SQXS was determined in the presence of its stress impurities. It was assessed by performing forced degradation studies on pure standards of the SQXS separately to indicate the initial results and on samples of sulfaquinoxaline sodium 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<sub>2</sub>O<sub>2</sub>). 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: Summary of the forced degradation study**

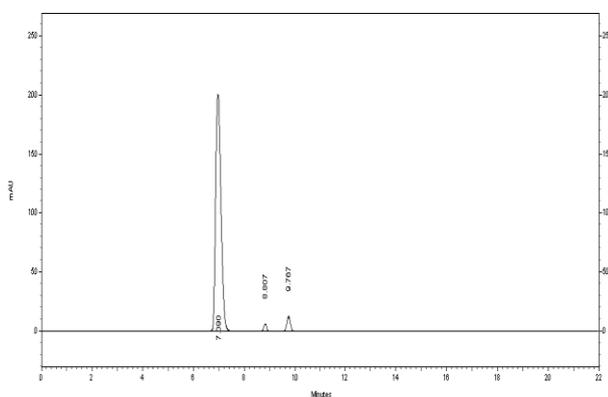
Name	Stress condition	Degradation%	Purity index*
SQXS standard	Acidic/0.10 N HCl / 60 min at RT	4.3	1.0000
	Alkaline/0.10 N NaOH / 60 min at RT	3.6	0.9997
	Oxidative/3.0% H <sub>2</sub> O <sub>2</sub> /24 hours at RT	36.7	0.9991
	Thermal/105 °C/72 hours	8.8	0.9998
	Light/ UV-254nm /36 hours	8.2	1.0000
SQXS sample	Acidic/0.10 N HCl / 60 min at RT	4.4	0.9999
	Alkaline/0.10 N NaOH / 60 min at RT	3.7	0.9998
	Oxidative/3.0 % H <sub>2</sub> O <sub>2</sub> /24 hours at RT	36.1	0.9994
	Thermal/105 °C/72 hours	9.0	1.0000
	Light/ UV-254nm /36 hours	8.3	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 2 revealed that the oxidative stress results showed extensive degradation in comparison to other stress conditions. Peak purity index for SQXS was found to be not less than 0.9991, a higher value than the accepted limit (0.990). Therefore, there was no interference between the SQXS peak and any other stress impurity peaks in the chromatogram. Almost the same pattern of degradation was obtained for SQXS in the Sulfaquinoxaline sodium water soluble powder samples. Figures (4-8) show the chromatographic profiles of the SQXS and the degradation products after exposing the Sulfaquinoxaline sodium water soluble powder to different stress conditions as in Table 2.



**Fig. 4: Chromatogram of SQXS water soluble powder upon exposure to UV-light**

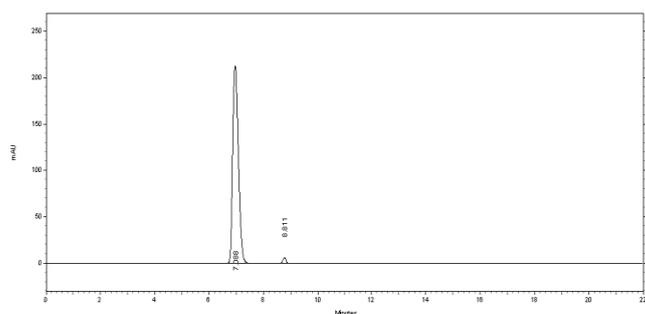


**Fig. 5: Chromatogram of SQXS water soluble powder upon exposure to heat**

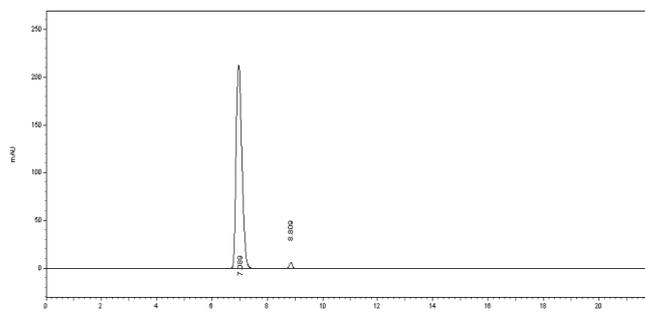
**Sensitivity**

The sensitivity of the method was explored via measuring the limit of detection (LOD) and the limit of quantitation (LOQ) for SQXS at a signal-to-noise ratio of 3 and 10 respectively. It has been achieved by injecting a series of SQXS diluted solutions with known

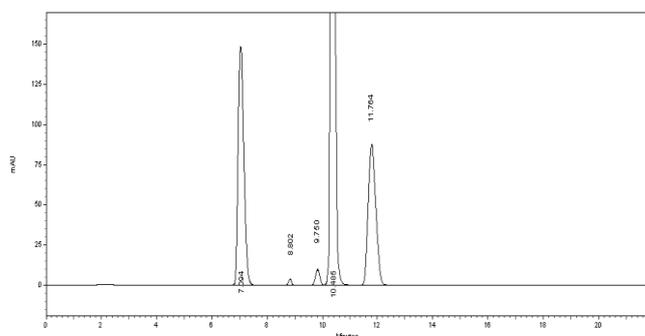
concentrations. LOD was found to be 0.04 µg/ml. LOQ was found to be 0.13µg/ml with RSD of 3.8% (accepted value is less than 10%).



**Fig. 6: Chromatogram of acidic degradation of the SQXS water soluble powder**



**Fig. 7: Chromatogram of basic degradation of the SQXS water soluble powder**



**Fig. 8: Chromatogram of oxidative degradation of the SQXS water soluble powder, the peak at 10.485 minutes is due to H<sub>2</sub>O<sub>2</sub>.**

**Linearity and range**

Different amounts of SQXS in the range of 50% to 150% of the labeled amount (5 concentration levels and 3 replicates each) were spiked to Sulfaquinoxaline sodium water soluble powder matrix

(Placebo). The linearity in the range of 12.5-37.5 µg/ml for SQXS 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<sup>2</sup> of 0.9998 (Fig.9, Table 3).

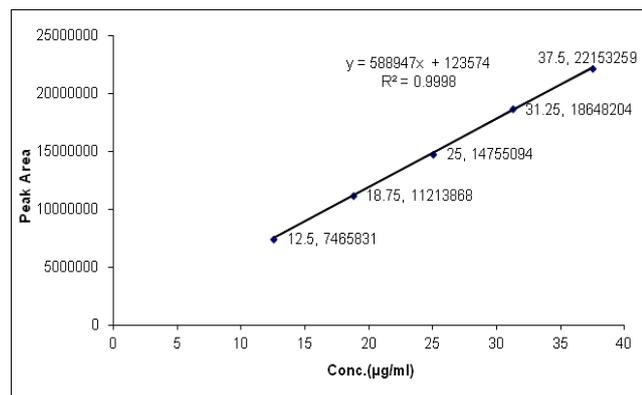


Fig. 9: Linearity and range for SQXS

Table 3: Regression statistics

Active ingredient	Linearity range (µg/ml)	(R <sup>2</sup> )	Linearity equation*	Y-intercept (%)
SQXS	12.5-37.5	0.9998	Y=588947X+123574	0.84%

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

**Accuracy (recovery)**

Accuracy was determined by the recovery study of known amounts of SQXS standard added to a placebo matrix of water soluble powder dosage form. Different concentrations of the SQXS were added to placebo matrix and the recovery was measured. The accuracy as reflected from recovery data of the SQXS is listed in Table 4. The average recovery data of SQXS showed results between 98.9% and 100.9% which are within the acceptable limit of (98.0 to 102.0% as set by the Palestinian Ministry of Health Registration Department criteria).

Table 4: Average recoveries, % RSD values at five concentration levels of spiking of SQXS

Active ingredient	Amount added (level %)	Average recovery (%) ± S.D (n=3)	RSD (%) (n=3)
SQXS	12.5 µg /ml (50%)	98.9 ± 0.41	0.41
	18.75 µg /ml (75%)	100.5 ± 0.87	0.87
	25.0 µg /ml (100%)	99.8 ± 1.12	1.12
	31.25 µg /ml (125%)	100.9 ± 0.76	0.75
	37.5 µg /ml (150%)	99.5 ± 0.97	0.97

**Repeatability**

One laboratory analyst carried out the assay of SQXS on six determinations of homogeneous sample of Sulfaquinoxaline sodium 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 SQXS revealed %RSD value of 0.77% 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 SQXS on twelve homogeneous samples of Sulfaquinoxaline sodium 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 SQXS revealed % RSD values of 1.46% which is within the acceptable limit of 2.0%. The results of the assay of the SQXS 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<sub>4</sub>AC solution concentrations in mobile phase (190mM, 200mM and 210mM) and different ACN percentages in mobile phase (88%, 90% and 92%) 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 SQXS showed results between 98.8% and 101.1% which are within the acceptable limit of (98.0 to 102.0%) (Table 5).

Table 5: Robustness testing of the SQXS active ingredient

Active ingredient	Parameter	Modification	Average assay%±S.D (n=3)
SQXS	Flow rate (ml/min)	0.45	100.4 ± 0.94
		0.50	100.2 ± 1.07
		0.55	99.7 ± 1.21
	Mobile phase ratio (v/v)	12:88	98.8 ± 0.88
		10:90	99.9 ± 1.14
		8:92	100.1 ± 1.21
	Temperature (°C)	22	99.3 ± 0.96
		25	100.3 ± 1.18
		28	99.6 ± 0.98
	NH <sub>4</sub> AC Conc.(mM)	190	99.7 ± 1.06
		200	100.5 ± 0.91
		210	99.1 ± 0.84
	Mobile phase pH	5.5	99.8 ± 1.27
		5.7	99.4 ± 0.97
		5.9	101.1 ± 0.73
Column batches	L010129977	99.4 ± 0.96	
	L010134877	100.3 ± 1.17	

**CONCLUSION**

The validated HPLC method developed for the quantitative quality control determination of Sulfaquinoxaline sodium 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 Sulfaquinoxaline sodium in its water soluble powder in the presence of excipients, SQXA and other degradation products. The assay showed complete separation of SQXS from SQXA, from other 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 Sulfaquinoxaline sodium water soluble powder.

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