Deanship of Graduate Studies

Al-Quds University

Validated HPLC Method to Simultaneously Determine Amprolium Hydrochloride, Sulfaquinoxaline Sodium and Vitamin K_3 in A.S.K Powder Manufactured by Pharmacare-PLC

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Thesis Approval

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Dedication

To my parents for their extended support and sincere praying.

To my beloved wife, without her patience and continuous encouragement this work would not have been possible.

To my beloved kid, Mohammad, who light my life up and give me the power to keep going.

To Pharmacare pharmaceutical company colleagues for their continuous support of equipments, materials and enthusiasm.

Declaration

This is to declare that this work has been done by the author and submitted to Al-Quds University to acquire the Degree of Master of Applied and Industrial Technology and all the results are due to my own research and this thesis (or part of it) has not been submitted for a higher degree to any other institution or university for any academic qualification.

M. Ghanem

Mashhour Mohammad "Farid Ismaei'l" Ghanem

Date: / /1432H

10 / 09 / 2011G

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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 simultaneous determination of Amprolium hydrochloride , Sulfaquinoxaline Sodium , and Vitamin K_3 (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 (NH_4AC) buffer and acetonitrile (ACN) (15:85; v/v) with pH adjusted to 5.7 by glacial acetic acid (G.A.A) 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 the separation and column efficiency were thoroughly investigated, including the concentration of the organic solvent (ACN) in the aqueous-organic mobile phase, the ionic strength of the NH_4AC buffer and pH of the mobile phase.

The optimized method was finally subjected to full analytical validation by examining specificity, accuracy, precision, linearity, range, ruggedness, robustness and stress conditions. The results were evaluated according to the International Conference on Harmonization (ICH) and United States Pharmacopoeia (USP33/NF28) guidelines and the method fulfilled validation criteria. In addition, the assay was shown to indicate stability and can be used to analyze for degradation products; for example; sulfaquinoxaline impurity A.

The developed method is sensitive, specific, fast, accurate and requires minimum sample manipulation. The analysis time requires only 18 minutes. The new optimized HPLC method was applied on three commercial A.S.K batches for analysis of amprolium hydrochloride, sulfaquinoxaline sodium and vitamin K_3. All the active ingredients were separated from excipients according to the authentic specifications.

Key words: Amprolium, Sulfaquinoxaline, Menadione sodium bisulfite, A.S.K Powder, ZIC-HILIC, validation, Stability-indicating.

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List of Abbreviations

Abbreviations	Definitions
PABA	Para-aminobenzoic acid
HPLC	High performance liquid chromatography
NP	Normal phase
RP	Reverse phase
IEX	Ion exchange
SEC	Size-Exclusion Chromatography
PIC	Paired ion chromatography
HILIC	Hydrophilic Interaction Liquid Chromatography
GLP	Good Laboratory Practice
ICH	International Conference on Harmonization
USP	United State Pharmacopoeia
NF	National Formulary
BP	British Pharmacopoeia
ZIC-HILIC	Zwitterionic Hydrophilic Interaction Liquid Chromatography
µs	Micro Siemens
TOC	Total organic carbon
cm	Centimeter
ppb	Part per billion
g	Gram
mg	Milligram
µg	Microgram
µl	Microliter
µm	Micrometer
M	Molar
mM	Millimolar
ml	Milliliter
i.d.	Internal diameter
S.D	Standard Deviation
RSD	Relative Standard Deviation

λ	Wavelength
nm	Nanometer
Max.	Maximum
NLT	Not less than
dp	Particle size diameter
pH	Negative logarithm of H^+ concentration
ACN	Acetonitrile
NH_4AC	Ammonium acetate
MeOH	Methanol
NaOH	Sodium hydroxide
H_2O_2	Hydrogen peroxide
Rs	Resolution
UV	Ultraviolet
%	Percent
HCl	Hydrochloric acid
T_F	Tailing factor
C.V.	Coefficient of variation
R^2	Regression coefficient
v/v	Volume per volume
n	Number of determinations
A.C.	Acceptance criteria
Av.	Average
PAV	Peak area value
Δ	Difference
C.I.	Confidence interval

Chapter One

Introduction

1.1 General Introduction

The three combined active pharmaceutical components to be separated and analyzed are mainly used for the treatment and control of coccidiosis.

> "**Coccidiosis** is an acute invasion and destruction of intestinal mucosa by protozoa of the genera Eimeria or Isospora. Infection is characterized by diarrhea, fever, inappetence, weight loss, emaciation, and sometimes death. Coccidiosis is a serious disease in cattle, sheep, goats, pigs, poultry, and also rabbits, in which the liver as well as the intestine can be affected. In dogs, cats, and horses, it is less often diagnosed but can result in clinical illness". [1]

Coccidiasis is the infection of animals with coccidia but without apparent clinical signs. Coccidiasis is much more prevalent than coccidiosis and is thought to result in poor feed efficiency under intensive rearing conditions. [1]

Anticoccidial drugs:

Many drugs are available for prevention and treatment of coccidiosis in chickens, and in turkeys. Anticoccidials are usually given to poultry in the feed to prevent acute disease and the economic loss often associated with subacute infection. [2] They can be used also for prophylactic purpose because most of the damage occurs before signs become apparent,

and delayed treatment may not benefit the entire flock. Water medication is better than feed medication for therapeutic treatment. Antibiotics and increased levels of vitamins A and K are sometimes added to the diet to improve rate of recovery and prevent secondary infections. [2]

Continuous use of anticoccidial drugs may result in selection for and survival of drug-resistant strains of coccidia. While there is little cross-resistance to anticoccidials with different modes of action, there is widespread resistance to most drugs. [3]

There are two distinct classes of anticoccidials: [4]

1.) Coccidiostats that inhibit growth of intracellular coccidia, giving rise to latent infection after drug withdrawal.
2.) Coccidiocides, which destroy coccidia during their development. Some anticoccidial drugs may be initially coccidiostatic but after long use become coccidiocidal. Most anticoccidials currently used in poultry production are coccidiocides. [4]

Many pharmaceutical veterinary products are available in the market for the treatment and control of coccidiosis and some contains several active ingredients such as Amprocoxin-silv (brand drug) and A.S.K powder (generic drug).

A.S.K Powder is a veterinary drug which is currently manufactured by Pharmacare pharmaceutical company in Palestine (See Table 1.1). It is a generic drug which is similar in composition to Amprocoxin-silv® that manufactured by Silvavet company for veterinary medicines.

A.S.K Powder is used in veterinary field for the following reasons:

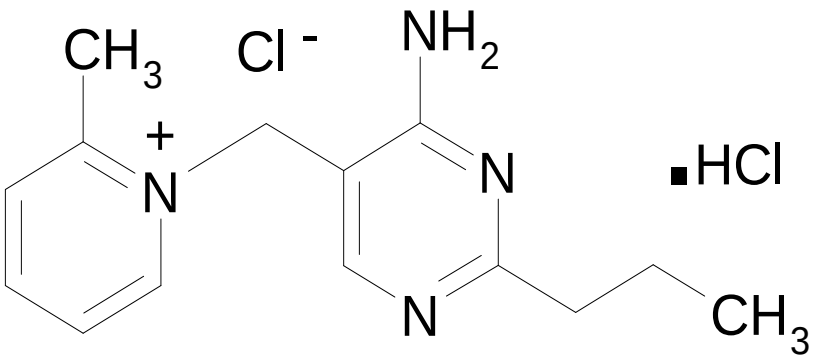
1) Prevention and treatment of coccidiosis in poultry, lambs, calves and pigs.
2) Control of coccidiosis and acute fowl cholera in chickens and acute fowl cholera and coccidiosis in turkeys, when caused by pathogens susceptible to sulfaquinoxaline.

Table (1.1): Pharmaceutical and clinical information of A.S.K Powder:

Product Name	A.S.K
Pharmaceutical Form	Powder
Groups	Anticoccidia
Packages	200g
Composition	Each 1g Contains: Amprolium HCl 200 mg Sulfaquinoxaline sodium 200 mg Vitamin K_3 2 mg
Indications	"Amprolium is considered as a common compound which is used as anti-coccidia in layer and hens mothers during fosterage period. It has very low poisoning level, its activity increases when it is associated with Sulfaquinoxaline. It has good effect against Eimeria tenella, Eimeria necatrix, medium effect against Eimeria acervuline and weak effect against Eimeria aximia and Eimeria brunetti. It is used to treat coccidia especially when coccidia accompanied with E. coli, salmonella and staphylococcus." [5]
Usage and Dosage	It is used with drinking water. 100g for each 200 liters of drinking water daily. [5] The treatment continues for 3 days then stopped for 3 days then resumed for 3 other days. [5]

Brief description of the drugs combination used in the study:

1.) Amprolium hydrochloride is 1-[(4-Amino-2-propyl-5-pyrimidinyl) methyl]-2-picolinium chloride monohydrochloride. ($C_{14}H_{19}ClN_4 \cdot HCl$; M.Wt. = 315.24).



It is white to light yellow powder, freely soluble in water, in methanol, in alcohol, and in dimethylformamide; sparingly soluble in dehydrated alcohol; practically insoluble in isopropyl alcohol, in butyl alcohol, and in acetone. [6]

Mechanism of action:

It is structurally related to vitamin thiamine and the antiparasitic activity of the drug is thought to be related to competitive inhibition of active thiamine transport into the parasite. [7]

Indications and clinical uses:

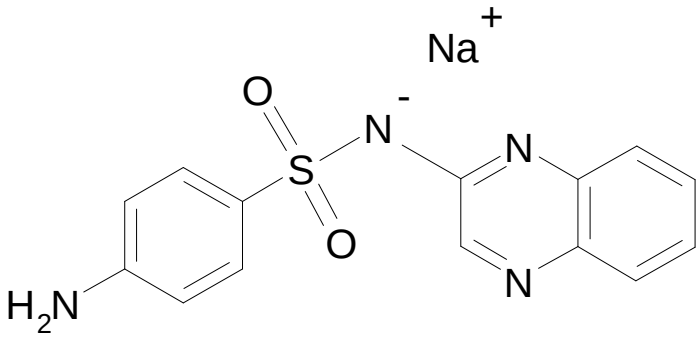
Amprolium is used to control and treat coccidiosis in calves, sheep, goats, puppies, and birds. It is administered orally, often mixed with food. [8]

Since amprolium has poor activity against some Eimeria spp , its spectrum has been extended by using it in mixtures with the folic acid antagonists, ethopabate and sulfaquinoxaline. [9]

Amprolium is thought to act primarily upon the first generation schizont in the cells of the intestinal wall, preventing differentiation of the metrozoites. It may suppress the sexual stages and sporulation of the oocysts. [10]

Amprolium is a thiamine analogue. Concurrent administration of thiamine can antagonize anticoccidial activity of Amprolium. [11]

2.) Sulfaquinoxaline sodium is N'1-Quinoxalin-2-ylsulphanilamide sodium salt ($C_{14}H_{11}N_4NaO_2S$; M.Wt. = 322.32)



It is white to yellow powder, solubility in H_2O: 50 mg/ml clear to slightly hazy, yellow to dark yellow solution. [12]

Mechanism of action:

Sulfaquinoxaline is a derivative of sulfanilamide (structurally similar to para-aminobenzoic acid) and it competes with para-aminobenzoic acid (PABA) for enzyme that synthesizes dihydrofolic acid in bacteria. [13]

Indications and clinical uses:

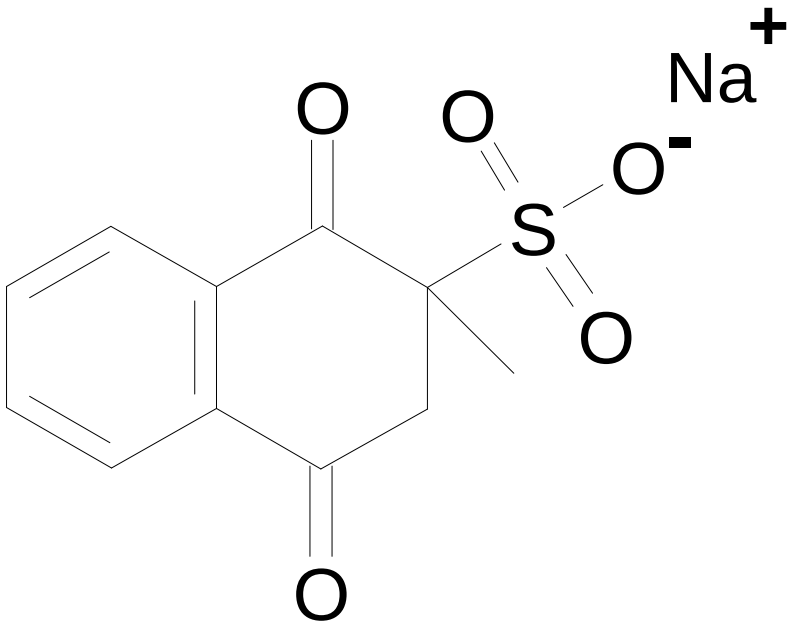
Sulfaquinoxaline is an oral baceriostatic antibiotic and coccidiostat. It is used for the prevention and treatment of intestinal coccidiosis and, in rabbits, hepatic coccidiosis due to Eimeria Stiedae. [14]

Chronic treatment with sulfquinoxaline leads to vitamin deficiency, especially vitamin K. [15]

> "For the use in poultry, Sulfaquinoxaline has been administered to control coccidiosis. Mathis and McDougald (1984) described the therapeutic effectiveness of sulfaquinoxaline and Sulfaquinoxaline-pyrimethamine against several coccidia species of Eimeria. It was determined from that study that both Sulfaquinoxaline and Sulfaquinoxaline-pyrimethamine were highly effective against E. acervulina but less effective against E. tenella. In addition, the potentiated mixture was determined to be more effective against E.tenella than Sulfaquinoxaline alone, although neither mixture was found to be particularly effective against any cecal coccidia.

Amprolium was found to be efficacious against cecal-dwelling forms of coccidia, hence amprolium has been combined with Sulfaquinoxaline or Sulfaquinoxaline-pyrimethamine to enhance the spectrum of activity. Ineffectiveness of Sulfaquinoxaline-pyrimethamine against E.tenella has also been documented in another study (chapman 1989), underlining the importance of correct cocciadia species identification before instituting anticoccidial therapy with sulfaquinoxaline or any other sulfonamide". [16]

3.) Vitamin K_3 sodium bisulfite (menadione sodium bisulfite) is 4-Naphthalenedione, 2-methyl-. 2-Methyl-1, 4-naphthoquinone ($C_{11}H_9NaO_5S$; M.Wt. = 276.24; CAS Registry Number: 130-37-0)



It is white to off-white powder; soluble in water (50mg/ml give clear colorless to light yellow solution). [17]

Menadione sodium bisulfate (Anti-blood) is a synthetic vitamin K compound used for the treatment of prolonged bleeding due to Vitamin K deficiency states. [18]

1.2 Modes of High Performance Liquid Chromatography (HPLC)

In the modern pharmaceutical industry, HPLC is still the major and integral analytical tool applied in all stages of drug discovery, development, and production. HPLC is one of the most important techniques in pharmaceuticals analysis, because of its speed, efficiency of separation, versatility, sensitivity, and its convenience for qualitative & quantitative analysis.

Chromatographic separations are based on a forced transport of the liquid mobile phase carrying the analyte mixture through the stationary phase. The differences in the interactions of the analytes with the surface of this stationary phase or the differences in the sizes and shape of the analytes resulting in different retention times for mixture components. [19]

There are different modes of liquid chromatographic methods. Our focus in this section is on partition chromatography in which there is a competition between two immiscible solvents; stationary phase and mobile phase. The main types of HPLC techniques are partition, ion-exchange (IEX), and size-exclusion (SEC). There are three basic types of molecular forces that can be involved in these as shown below: [20]

1. Polar forces are the dominant type of molecular interactions employed in NP HPLC.
2. Dispersive forces are employed in RP HPLC
3. Ionic forces are employed in IEX HPLC

The fourth type of HPLC technique, SEC HPLC is based on the size and shape of analyte separated.

1.2.1. Ion-Exchange chromatography (IEX):

The separation in this method is based on the different affinities of the analyte ions for the oppositely charged ionic centers in the stationary phase or adsorbed counterions in the hydrophobic stationary phase. Depending on the charge of the exchange centers on the

surface of the stationary phase, the resin could be either anion-exchanger (positive ionic centers on the surface) or cation-exchanger (negative centers on the surface). [21]

1.2.2. Partition Chromatography or Liquid/liquid Chromatography (LLC):

The stationary phase is an immobilized liquid upon an inert and porous material. [22] This technique can be subdivided into liquid-liquid and bonded phase chromatography. The main difference is the method by which the stationary phase in helded on the support particles of the packing. In the former, the stationary phase is retained on the surface of the support by physical adsorption while in the later, it is bonded chemically. Bonded phase is more popular nowadays due to the more stability of the stationary phase. [23]

The separation in this technique depends upon the distribution of sample components between two immiscible phases, according to their relative solubilities. The stationary phase being held in a column in the form of thin form on the support, and the mobile phase being forced through the column carrying the sample components along. To avoid mixing of the two phases the two partitioning liquids must differ greatly in polarity.

The degree of interaction of analytes with the mobile phase and the stationary phase whether normal or reversed, affects the retention time of the analytes. In principle, the polarity of the stationary phase can lead to the following situations: [24]

Normal phase HPLC: in this mode, the stationary phase is a relatively polar and the mobile phase is a nonpolar. [24] Retention occurs through polar interactions, such as hydrogen bonding and dipole interactions, between the solute and the stationary phase. [25]

Reversed-phase HPLC: in this mode, the stationary phase is a relatively nonpolar and the mobile phase is a polar, the polar components favor the mobile phase and elute faster. [24]

RP chromatography is the preferred HPLC mode in the pharmaceutical industry due to its mobile-phase compatibility with the typical polar drug substance, the higher efficiencies associated with this mode, shorter re-equilibration times, and the ability to run gradient methods covering a large range in polarity. [26]

Paired-ion chromatography (PIC) or Ion-pair chromatography is a technique related to RP-HPLC that are established for the separation of highly polar compounds such as amino acids and organic acids that do not adhere at all to the stationary phase if it is nonpolar . Thus this polar character must be reduced to increase retention. To modify the ionic charge a strongly ionic reagent, called a ion pairing reagent, is added to the mobile phase. This is usually a compound with a carbon chain (weakly polar) possessing a functionality whose charge is opposite to the analyte to be separated (e.g. heptane sulfonic acid if the ionic solute is a base). This forms a 'neutralized' ion pair, a less polar species, fairly stable and lipophilic, which will initially stick to non polar stationary phase but can still be eluted, apparently by a type of ion-exchange mechanism. [27] So to make the ionic compound have sufficient lipophilic character to be retained by the column, a counter ion (pairing ion) is added to the eluent. The sample ion, x^{-n}, plus the counter ion, Q^{+n}, combine to form an ion pair ($x^{-n}\ Q^{+n}$) which has sufficient affinity for the reverse phase column to be retained by it . [28]

The use of PIC is not always preferable since a thorough washing of the column is needed after separation. The reproducibility of the column however is not stable.

Another practical alternative is to use relatively new column technology, the HILIC chromatography.

1.2.3. Hydrophilic Interaction Liquid Chromatography (HILIC):

Another option to conventional NP chromatography is hydrophilic interaction chromatography (HILIC). This technique utilizes a polar stationary phase with aqueous/organic mobile phase but with very high percentages of organic modifier. A simple acetonitrile/aqueous buffer mobile phase is commonly utilized in conjunction with a silica or amino stationary phase. The most common used buffer salt is the ammonium acetate because it possesses good solubility at high organic content.

An adsorbed water layer on the silica substrate is formed under these chromatographic conditions. Polar solutes partition from the highly organic bulk mobile phase into the adsorbed water layer where they can undergo polar interactions. In addition, positively

charged solutes, such as amines, can undergo ionic interactions with charged silanol groups. As a consequence, retention of solutes increases with their increasing polarity. This mode is particularly useful for the separation of very polar solutes and/or raw materials that show minimal or no retention under RP conditions and are very strongly retained under NP conditions. [29]

1.3 HPLC Analytical Method Validation

GLP requires that any chromatographic method, which is used to assess compliance of the pharmaceutical product with established specification, must meet proper standard of accuracy and reliability. A validated method can give consistent and reliable results. The validation is mainly concerned with source of errors and their estimation in the experimental results. If the estimated errors are within the acceptance limit, then the method is qualified for its intended use.

When analytical methods need to be validated or revalidated?

1.) Before introduction of a new method into routine use.
2.) Whenever the conditions are changed to which the method has been validated (e.g., an instrument with different characteristics or sample with a different matrix), and
3.) Whenever the method is changed and the change is outside the original scope of the method.

Analytical validation should be performed as soon as possible after development of the method, and must be completed before the results are used for registration or product release.
Validation should cover all stages through sample selection and preparation, analyte recovery, suitability of equipment, and the assessment, interpretation and reporting of results.

Validation of methods for the quantitative analysis of drugs involves determining as minimum, their specificity, linearity, working range, accuracy and precision under the conditions and with the typical sample matrices that will be met in practice.

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for the intended analytical applications**.** [30]

Types of Analytical Procedures to be validated: [31]

The discussion of the validation of analytical procedures is directed to the four most common types of analytical procedures:

• Identification tests.
• Quantitative tests for impurities' content.
• Limit tests for the control of impurities.
• Quantitative tests of the active moiety in samples of drug substance or drug product

A brief description of the types of tests considered above is provided below:

• Identification tests are intended to ensure the identity of an analyte in a sample. This is normally achieved by comparison of a property of the sample (e.g., spectrum, chromatographic behavior, chemical reactivity, etc) to that of a reference standard.
• Testing for impurities can be either a quantitative test or a limit test for the impurity in a sample.
• Assay procedures are intended to measure the analyte present in a given sample. In the context of this document, the assay represents a quantitative measurement of the major component(s) in the drug substance.

Typical validation characteristics which should be performed are listed in table (1.2).

Table (1.2): Typical validation characteristics which should be performed according to ICH/USP33 guidelines [32]

Analytical Performance Characteristics	Assay	Identification	Impurity quantitative	Impurity qualitative
Specificity	Yes	Yes	Yes	Yes
Linearity	Yes	No	Yes	No
Range	Yes	No	Yes	No
Accuracy	Yes	No	Yes	No
Precision -Repeatability -Intermediate precision	 Yes Yes	 No No	 Yes Yes	 No No
Detection Limit	No	No	Yes /No	Yes
Quantitation Limit	No	No	Yes	No
Robustness	Yes	Yes	Yes	Yes

> **"Analytical Procedure**: The analytical procedure refers to the way of performing the analysis. It should describe in detail the steps necessary to perform each analytical test. This may include but is not limited to: the sample, the reference standard and the reagents preparations, use of the apparatus, generation of the calibration curve, use of the formulae for the calculation,... etc. **" [33]**

"Specificity: it is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc". **[33]** Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s). **[33]**

"Linearity: The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.**" [33]**

> **"Range:** The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity." [33]

"Accuracy: The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found." [33]

> **"Precision:** The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility." [33]

"Repeatability: The precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision." [33]

"Intermediate precision: The precision within-laboratories variations: different days, different analysts, different equipment, etc." [33]

"Detection limit: The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value." [33]

The detection limit (DL) can be determined based on the standard deviation of the response and the slope as expressed in the below formula:

$$DL = (3.3 \times S.D) / Slope$$

S.D: The standard deviation of the response

Slope: The slope of the calibration curve

"Quantitation limit: The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products."[33]

The quantitation limit (QL) can be determined based on the standard deviation of the response and the slope as expressed in the below formula:

$$QL = (10 \times S.D) / Slope$$

S.D: The standard deviation of the response

Slope: The slope of the calibration curve

"**Robustness:** Measure of an analytical procedure's capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage." [33]

1.4 Research Problem

Pharmacare pharmaceutical company has developed a new generic veterinary product under the name of A.S.K Powder. Up to now, this product can not be registered at the Ministry of Health in Palestine since there is no quality control method in the official pharmacopoeias or any other published validated method that enable us to analyze the drug combination simultaneously and perform stability studies including accelerated and long term stability studies which are the major requirements for registration of any pharmaceutical drug in Palestine or other countries.

1.5 The Objectives of this Research Project

General objective:

To develop an authentic validated quality control HPLC method to simultaneously analyze amprolium hydrochloride, sulfaquinoxaline sodium and vitamin K_3 (menadione sodium bisulfite) in A.S.K Powder and perform stability studies that enable us to register the drug at the Ministry of Health in Palestine.

Specific objectives:

1.) Since there is no HPLC assay that applies to analyze the pharmaceutical combination of amprolium hydrochloride, sulfaquinoxaline sodium and vitamin K_3 salt ingredients together this triggered us to develop a specific, fast and accurate HPLC method that allows the simultaneous separation within a reasonable retention time.

2.) To validate the new developed method according to ICH and USP guidelines and specifications. The validated method will serve as authentic quality control method to analyze A.S.K Powder and perform the stability of the drug and thus register the drug in Palestine or in any other country in the future.

3.) To publish this validated method in the British pharmacopoeia (BP) or United States pharmacopoeia (USP) as an official method for analysis of the three active components simultaneously.

1.6 Research Hypothesis

Hydrophilic interaction liquid chromatography (HILIC) is a relatively new technique that is designed for separation of very polar and hydrophilic compounds that may elutes with the void peak in the reversed phase system and solutes that have little or no retention on RPLC columns generally experience strong retention on HILIC columns. [34]

The HILIC technique thus bears similarities with traditional NPLC, but with the important difference that HILIC employs semi-aqueous mobile phases. Consequently, with respect to analyte solubility in the eluent and matrix compatibility, HILIC is superior, as the mobile phase compositions used are comparable to RPLC separations. Typical eluents for HILIC consist of 40-97% acetonitrile in water or a volatile buffer. [34]
Besides, HILIC is a very mass spectrometry (MS) friendly technique, and by changing from RPLC to HILIC a 10-1000 fold increase in sensitivity is often observed for hydrophilic analytes. Ion-pair reagents are also completely avoided, which is advantageous for preparative chromatography.[34]

ZIC-HILIC columns contain a stationary phase that is hydrophilic and quite often also charged (Figure 1.1). Hydrophilic compounds are generally more strongly retained on the column by interacting with the stationary phase thus achieving separation. [35]

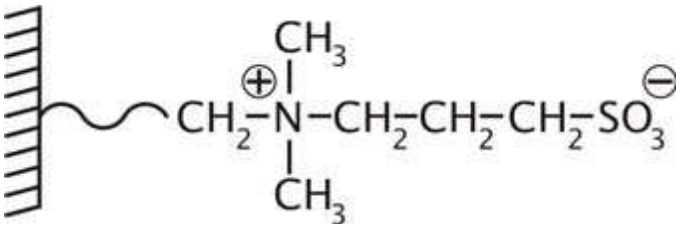


Figure (1.1): The functional group of the ZIC®-HILIC [34]

Under HILIC conditions, a water-enriched liquid layer will be established within the stationary phase. The separation is achieved by partitioning of solutes from the eluent into this hydrophilic environment (see Figure 1.2). Hence, both hydrogen bonding and dipole-dipole interactions are factors governing retention. The retention will also be influenced by

electrostatic (ionic) interactions, as illustrated in Figure 1.2 for a zwitterionic stationary phase. [35]

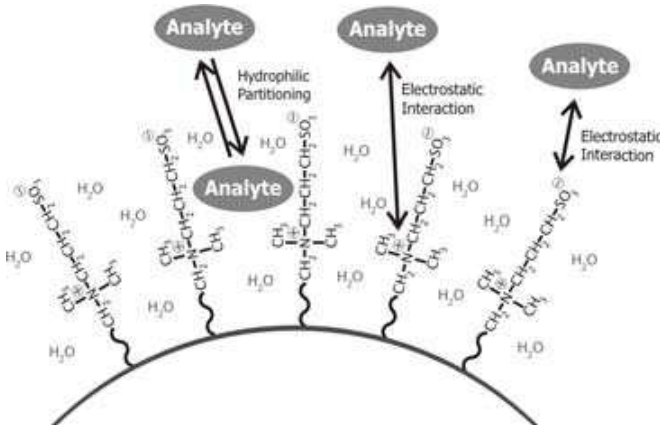


Figure (1.2): The retention processes in HILIC illustrated by hydrophilic partitioning, and electrostatic interactions with either positive or negative charges.[35]

The permanently charged, yet overall neutral, highly polar zwitterionic ZIC®-HILIC material provides a unique environment, not only particularly capable of solvating polar and charged compounds, but also offering the possibility of weak electrostatic interaction with analytes carrying either positive or negative charges. The retention thus generally increases with hydrophilicity and with charge of the analyte, and the selectivity can be tuned while maintaining a low ionic strength. These properties make the ZIC®-HILIC material excellent for separations of a wide range of polar and hydrophilic compounds.

The silica-based ZIC®-HILIC stationary phase carries a covalently bonded, permanently zwitterionic, functional group of the sulfobetaine type, see Figure 1.1.[36]

Chapter Two

Literature Review

The assays of combinations of active ingredients should be specific, and sensitive enough to analyze the active ingredients in pharmaceutical formulations without interferences. The method should also be fast, with a minimum sample manipulation, and of stability-indicating capability.

The three drugs that are used as a mixture in this veterinary combination need a fast HPLC method to separate and quantitate them simultaneously. An extensive literatures survey using ScienceFinder (the largest and most comprehensive database) and the official pharmacopoeias did not reveal any method for the simultaneous separation, and assay determination of these components mixture in the drug.

However, there are different non-chromatographic and chromatographic particularly RP-HPLC and ion-pair RP-HPLC methods of analysis that have been applied to assay these compounds individually or when present in combinations with other ingredients:

The official BP2007 assay method for the quantitative determination of amprolium hydrochloride has been based on non-aqueous titration which is accurate, simple and rapid but it applies only to amprolium hydrochloride when present as active pharmaceutical ingredient and is not of stability-indicating nature. [37]

The official USP33/NF28 assay method for quantitative determination of amprolium hydrochloride has been based on HPLC, which is accurate, specific and simple but it applies only to amprolium hydrochloride present as active pharmaceutical ingredient. This method is based on reversed phase, ion-pair HPLC technique using sodium 1-heptanesulfonate , water, glacial acetic acid, triethylamine, methanol (MeOH), and ACN as a mobile phase and a 4.6-mm × 25-cm column containing trimethylsilane chemically bonded to porous silica particles, 3 to 10 µm in diameter. [38]

The official USP33/NF28 assay method for quantitative determination of amprolium hydrochloride has been based on HPLC, which is accurate, simple and specific but it applies only when the amprolium hydrochloride is the only active ingredient in pharmaceutical formulations. Again, this method is based on reversed phase, ion-pair HPLC technique using sodium 1-hexanesulfonate, water, MeOH, and ACN as mobile phase and a 3.9-mm × 30-cm column that contains phenyl groups chemically bonded to porous silica particles, 1.5 to 10 µm in diameter. [39]

The official BP2007 assay method for quantitative determination of sulfaquinoxaline has been based on titration which is accurate, fast and simple but it applies only to sulfaquinoxaline when present as active pharmaceutical ingredient and is not of stability indicating nature. [40]

The official USP33/NF28 assay method for quantitative determination of sulfaquinoxaline has been based on HPLC, which is accurate, simple and fast but it applies only when the sulfaquinoxaline is present as active pharmaceutical ingredient or is the only active ingredient in the pharmaceutical preparations. This method is based on reversed phase HPLC technique using monobasic ammonium phosphate, water, ACN, glacial acetic acid, tetrahydrofuran, and ammonium hydroxide as mobile phase and a 4-mm × 25-cm column that contains octadecyl silane chemically bonded to porous silica particles, 1.5 to 10 µm in diameter. [41]

The official USP33/NF28 assay method for quantitative determination of menadione has been based on oxidation-reduction titration, which is accurate, and fast but it applies only

to menadione present as an active pharmaceutical ingredient and needs chemical manipulation. The method is not of stability-indicating nature. [42]

The official USP33/NF28 assay method for quantitative determination of menadione has been based on ultraviolet/visible spectrophotometric method which leads to extensive interferences when used in the drug combination of A.S.K Powder. The method applies only when the menadione is the only active ingredient in the dosage forms and is not of stability-indicating nature. The method is time consuming and requires some chemical manipulation. [43]

The official BP2007 assay method for quantitative determination of menadione has been based on oxidation-reduction titration method, which is accurate, simple and fast but it applies only when the menadione is present as active pharmaceutical ingredient and need some chemical manipulation. [44]

Cheng, Huang, Chang, Yao, and Hwang (2008) have developed an HPLC method for the selective separation of sulfapyridine (SPD) from sulfaquinoxaline (SQX). The method was carried out with molecularly imprinted polymer (MIP) as the stationary phase and 34mmol/ml sodium dihydrogen phosphate buffer solution /ACN (3/2, v/v) as mobile phase under 272 nm UV detection. [45]

Lakkanatinaporn and Matayatsuk (2004) have developed a simple HPLC method for the separation and determination of sodium trimethoprim phenylpropanol disulphonate and sodium sulfaquinoxaline in veterinary preparations. Both drugs were separated well on a Kromasil C_{18} column (5 µm, 150 × 4.6 mm) using a mixture of ACN and 0.5% triethylamine in 1% acetic acid, pH 3 (18:82, v/v) as the mobile phase at the flow rate of 1.5 ml/min. The presence of both substances was monitored by UV absorption detection at 271 nm. [46] The method is accurate, specific, precise and fast.

Cruz, Soares, Marquesb and de Aquino-Netoa (2002) have developed an HPLC-electrospray ionization - tandem mass spectrometry (LC-ESI-MS/MS) to simultaneous determination of ten sulfonamide (SAs) residues (sulfacetamide, sulfathiazole, sulfamerazine, sulfamethazine, sulfamethoxypyridazine, sulfamethizole,

sulfachloropyridazine, sulfamethoxazole, sulfadimethoxine and sulfaquinoxaline) in eggs. Samples were extracted with acetonitrile and the supernatants were cleaned up by solid phase extraction. After solvent evaporation and reconstitution, aliquots of the final extracts were injected into a LC-ESI-MS/MS.

The mobile phase used was ultrapure water (A) and ACN (B), both with 0.1% of formic acid, with a constant flow of 1 ml min-1. Gradient elution started with 100% of A, then decreased to 50% in 10 min, returning to 100% in 1 min and remaining in 100% for 1 min. The column used was a reversed phase column C_{18} 150 × 4.6 mm, 5 mm, X-Terra, Waters (Milford, USA). [47] This method requires extensive extraction which leads in many cases to non-reproducibility and requires trained technicians and it is very costly.

Kishida (2001) has developed a simple HPLC method for routine monitoring of seven residual sulfonamides (sulfadiazine (SDZ), sulfamerazine (SMR), sulfadimidine (SDD), sulfamonomethoxine (SMM), sulfamethoxazole (SMX), sulfadimethoxine (SDM), and sulfaquinoxaline (SQ)) in milk. The HPLC method was done on RP-4 column and 25 %(v/v) aqueous ethanol solution as mobile phase. Eluate was monitored using a photodiode array detector set at 269 nm. The method has been applied only after the sample was cleaned up by using an Ultrafree®-MC/PL centrifugal ultrafiltration unit. The average recoveries of each active were more than 82% and the HPLC analysis time is below 22 minutes. [48]

This method requires extensive extraction which leads in many cases to non-reproducibility and requires trained technicians.

Kozhanova, Fedorova, and Baram (2000) have developed a simple HPLC method for the separation and determination of Water- and Fat-Soluble Vitamins in Multivitamin Preparations. The method is capable of determination of vitamins B_1 and B_2, nicotinic acid, nicotinamide, pantothenic acid, folic acid, vitamins B_6, B_{12}, K_3, H, D_2, and D_3, and vitamin A and E acetates in multivitamin preparations using two chromatographic procedures. The vitamins were separated on column with the Nucleosil 100-5 C_{18} reversed-phase sorbent and use gradient elution mode for water soluble vitamins and the isocratic mode for fat-soluble vitamins .The presence of these vitamins were monitored by UV absorption detection at different wavelength. [49] The method is accurate, fast but need some sample manipulation and trained technicians.

Ya-Min Kao , Mei-Hua Chang, Chieu-Chen Cheng And Shin-Shou Chou (2000) have developed an HPLC method for the residual determination of 13 veterinary drugs, including clopidol, sulfadiazine, sulfathiazole, carbadox, sulfamerazine, ormethoprim, sulfamethazine, furazolidone, sulfamonomethoxine, sulfamethoxazole, ethopabate, sulfaquinoxaline, and sulfadimethoxine in chicken and swine muscles. Test samples were extracted with acetonitrile and filtered. The filtrate was partitioned with ACN-saturated n-hexane for removing the interference. After evaporation to dryness, the residue was passed through a Sep-Pak C18 cartridge for sample cleanup prior to HPLC analysis. The HPLC method was equipped with a photodiode array detector using a C18 (25 cm× 4.6 mm i.d., 5 μm) analytical column and a gradient elution of ACN and 0.05M sodium dihydrogen phosphate. **[50]**

However, the extraction and purification of these methods involves numerous and varying analytical steps which are labor intensive and time consuming and needs trained technicians.

Filimonov, Zamuraev, Balyatinskaya, and Kolosova (1999) have developed HPLC method for the determination of the synthetic fat-soluble vitamins including vitamins E acetate, K_3, D_2 in Hexavet, Undevit, and Hendevit multivitamin pharmaceutical preparations. This method is based on normal phase HPLC technique using hydroxylated silica gel column (100 ×5.4 mm) and n-hexane with polar solvents additives (aliphatic alcohols, dichloromethane, or 1,2-dichloroethane) as a mobile phase and monitored at UV absorption wavelength of 254 nm. The method is accurate, simple and fast. **[51]**

Boulaire, Bauduret, and Andre (1997) have developed an HPLC method with Matrix solid phase dispersion extraction technique for the isolation and determination of 14 veterinary drug residues (sulfathiazole, STH; sulfamerazine, SMR; sulfachloropyridazine, SCP; sulfamethazine, SMT; sulfamethoxypyridazine, SMO; sulfamethoxazole, SMA; sulfaquinoxaline, SQX; sulfadimethoxine, SDX; thiabendazole; mebendazole; nicarbazin; chloramphenicol ; furazolidone and Virginiamycin) in meat . Matrix solid phase dispersion (MSPD) was chosen as the extraction technique for this screening method. After extraction reversed-phase C18 column was used for the high performance liquid chromatography (HPLC) to separate the analytes with gradients of NH_4AC buffer-ACN-MeOH as mobile

phase. Photodiode array and fluorimetric detectors were used for this analysis. However, the extraction and purification of these methods involves numerous and varying analytical steps which are labor intensive and time consuming and needs trained technicians. [52]

Tan, Ramachandran 2, and Cacini (1996) have developed HPLC method for the assay of mixtures of amprolium and ethopabate in chicken feed utilizing reversed-phase high-performance liquid chromatography (HPLC). HPLC was done on a C-8 column with MeOH-water 40:60, containing octanesulfonic acid, triethylamine and acetic acid, as mobile phase. Eluate was monitored at 274 nm. [53]

The method has been applied only after the sample is extracted through solid phase extraction by passing methanol followed by distilled water and then treatment with HCl, ammonium hydroxide solution and finally centrifugation. This method is time consuming and there is possibility of active ingredients loss during the extraction, washing and centrifugation steps.

Ralph Hindle has developed a simple HPLC method for the analysis of sulfonamide antibiotics in pork muscle. The HPLC method was done on Zorbax XDB-C8,150mm ×4.6 mm,5µml with gradients of 0.1%formic acid in water (solvent A) and 0.1%formic acid in acetonitrile (solvent B) as mobile phase. The method is based on HPLC Mass spectroscopy using chemical ionization. [54]

The method has been applied only after the samples were extracted with acidified methanol and centrifuged. This method requires extensive extraction which leads in many cases to non-reproducibility and requires trained technicians.

Chapter Three

Experimental Part

3.1 Chemicals and Reagents

Ammonium acetate extra pure (Merck, Germany)
Acetic acid (Glacial) 100% (Merck, Germany)
Methanol HPLC grade (Merck, Germany)
Acetonitrile HPLC grade (Merck, Germany)
Hydrochloric acid fuming 37% (Merck, Germany)
Sodium hydroxide pellets GR for synthesis (Merck, Germany)
Hydrogen peroxide 30% for analysis (Merck, Germany)
Amprolium hydrochloride reference standard 99.4% (Lot no: SZBA068X) were purchased from Sigma-Aldrich (Germany)
Sulfaquinoxaline sodium reference standard 99.9% (Lot no: BCBB7740V) were purchased from Sigma-Aldrich (Germany)
Menadione sodium bisulfite reference standard 98% (Lot no: 048K1372) were purchased from Sigma-Aldrich (Germany)
Purified water (0.45 µm, Conductivity: Maximum 1.3µs/cm, TOC: Maximum 500 ppb).
A.S.K Powder samples were supplied by Pharmacare pharmaceutical company
All active ingredients and excipients usually used in manufacturing the pharmaceutical combination, were kindly supplied by Pharmacare pharmaceutical company

Hexane-1-sulfonic acid sodium salt for ion pair chromatography LiChropur (Merck, Germany)
Decane-1-sulfonic acid sodium salt (Merck, Germany)
Triethylamine (Merck, Germany)
Ammonia solution 25% for analysis, (Merck, Germany)

3.2 Apparatus and Laboratory equipment

Ultraviolet/visible spectrometer (PG Instruments, United Kingdom)
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)
Laboratory glassware (volumetric flasks, measuring cylinders, beakers, volumetric pipettes, and graduated pipettes)
Disposable Syringe 5ml, (Shandong Zibo shanchuan medical instruments CO., Ltd., China)
Syringe filters (Nylon $_{66}$) pore size 0.45µm, diameter 25mm (Axiva Sichem Biotech, India).
Magnetic stirrer model F-13 (Fried Electric, Haifa)
Mettler Toledo pH meter MP230 (Sigma-Aldrich, Germany)
Sonicator (Branson Ultrasonics Corporation, USA)
Vacuum pump & filtration assembly (NDS Technologies.Inc, Vineland)
Weighing balance (Ohaus, USA)

3.3 Analytical procedure

3.3.1. Chromatographic conditions:

The column utilized was a ZIC®-HILIC column (25.0 cm long ×4.6 mm i.d.) with particle size of 5 µm (Merck, Germany) protected with a ZIC®-HILIC guard column (20mm× 2.1mm, 5µm). The guard column was used to prolong the life time of the column and to avoid column blockage by any particulate matter.

The 0.2 M Ammonium acetate was prepared by dissolving 3.08 g of NH_4AC in purified water and diluted to 200 ml with the same solvent.

The optimum mobile phase finally selected for the assay was prepared by mixing 0.2 M NH_4AC and ACN (15:85; v/v), shaken well and left tell the temperature of the mobile phase reached to the room temperature. Then pH was adjusted to 5.7 by glacial acetic acid. The mobile phase was filtered using 0.45 µm microporous filters and was degassed by sonication prior to use. The standard and sample solutions were filtered using 0.45 µm membrane filters to prolong pump life and to avoid column blockage by any particulate matter.

The wavelength of 263 nm was chosen because it was found most appropriate for the three compounds simultaneously. The flow rate was 0.5 ml/minute as recommended by the column manufacturer whereby minimum height equivalent to theoretical plates and maximum number of theoretical plates are 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.

3.3.2. 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 K_3) in 80.0 ml of 90% ACN and diluting to 100.0 ml with the same solvent. 5 ml of this solution was diluted to 50 ml with 90% ACN. This brings about a concentration of 20.0µg/ml.

Standard solution for amprolium HCl, sulfaquinoxaline sodium and vitamin K_3 was 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 the diluted to 100.0 ml with 90% ACN. Then 5 ml of this solution was diluted to 50 ml with mobile phase. The solution

was filtered using 0.45 µm membrane filters. Final concentration was 20 µg/ml for amprolium hydrochloride, 20 µg/ml for sulfaquinoxaline sodium and 0.20µg/ml for vitamin K_3. This solution has been used within 24 hours if kept at 15 °C and protected from light.

3.3.3. 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 to 100 ml with the same solvent. Then 5 ml of this sample solution was diluted to 50 ml with mobile phase. The solution was filtered using 0.45 µm membrane filters. Final concentration was 20 µg/ml for amprolium hydrochloride, 20 µg/ml for sulfaquinoxaline sodium and 0.20µg/ml for vitamin K_3. This solution has been used within 24 hours if kept at 15 °C and protected from light.

3.3.4. Calculations:

Since the validation results indicated that the peak areas were linearly proportional to concentrations over a range of 12 µg/ml to 26µg/ml for amprolium hydrochloride, 12 µg/ml to 26µg/ml for sulfaquinoxaline sodium and 0.12 µg/ml to 0.26µg/ml for vitamin K_3 and also, as a consequence of linearity relationship, the amounts injected were always within the ranges of linearity found for these three compounds, the results were calculated using the following formulas:

$$\%\text{Label claim of Amprolium HCl} = \frac{Psa. \times Cst.}{Pst. \times Csa.} \times P \times \frac{100-W}{100}$$

Where;

Psa.: Average area of peak corresponding to amprolium hydrochloride in sample solution chromatograms.

Pst.: Average area of peak corresponding to amprolium hydrochloride in standard solution chromatograms

Cst.: Concentration of amprolium HCl in the standard preparation (µg /ml)= 20µg /ml

Csa.: Concentration of amprolium HCl in the sample preparation (µg/ml)= 20µg /ml

P: % assay of amprolium hydrochloride working standard (Anhydrous basis)

W= Water content of working standard

$$\text{-\%Label claim of Sulfaquinoxaline Sodium} = \frac{Psa. \times Cst.}{Pst. \times Csa.} \times P \times \frac{100-W}{100}$$

Where;

Psa.: Average area of peak corresponding to sulfaquinoxaline sodium in sample solution chromatograms.

Pst.: Average area of peak corresponding to sulfaquinoxaline sodium in standard solution chromatograms

Cst.: Concentration of sulfaquinoxaline sodium in the standard preparation (µg /ml)= 20µg /ml

Csa.: Concentration of sulfaquinoxaline sodium in the sample preparation (µg/ml)= 20µg /ml

P: % assay of sulfaquinoxaline sodium working standard (Anhydrous basis)

W= Water content of working standard

$$\text{-\%Label claim of Menadione} = \frac{Psa. \times Cst.}{Pst. \times Csa.} \times P \times (172.18/276.24) \times \frac{100-W}{100}$$

Where;

Psa.: Average area of peak corresponding to menadione in sample solution chromatograms.

Pst.: Average area of peak corresponding to menadione in standard solution chromatograms

Cst.: Concentration of menadione sodium bisulfite in the standard preparation (µg /ml) = 0.32µg /ml

Csa.: Concentration of menadione in the sample preparation (µg/ml)= 0.20µg /ml

P: % assay of menadione sodium bisulfite working standard (Anhydrous basis)

W= Water content of working standard

172.18 and 276.24 are the molecular weights of menadione and menadione sodium bisulfite respectively.

Chapter Four

Results and Discussion

4.1 Development and Optimization of the HPLC Method

Prior exploration of the best chromatographic conditions, the selection of an appropriate wavelength for all the compounds involved is a necessity. The overlaid ultraviolet absorption spectra for solutions of amprolium hydrochloride dissolved in mobile phase reveals two maxima namely 232 and 267nm, for sulfaquinoxaline sodium reveals two maxima at 249 and 263nm while for menadione sodium bisulfite reveals two maxima at 231 and 260 nm . As shown from figure 4.1, we noticed that the three compounds almost shared a wavelength near to 263 nm, therefore, 263 nm was chosen as the most appropriate wavelength throughout the investigations.

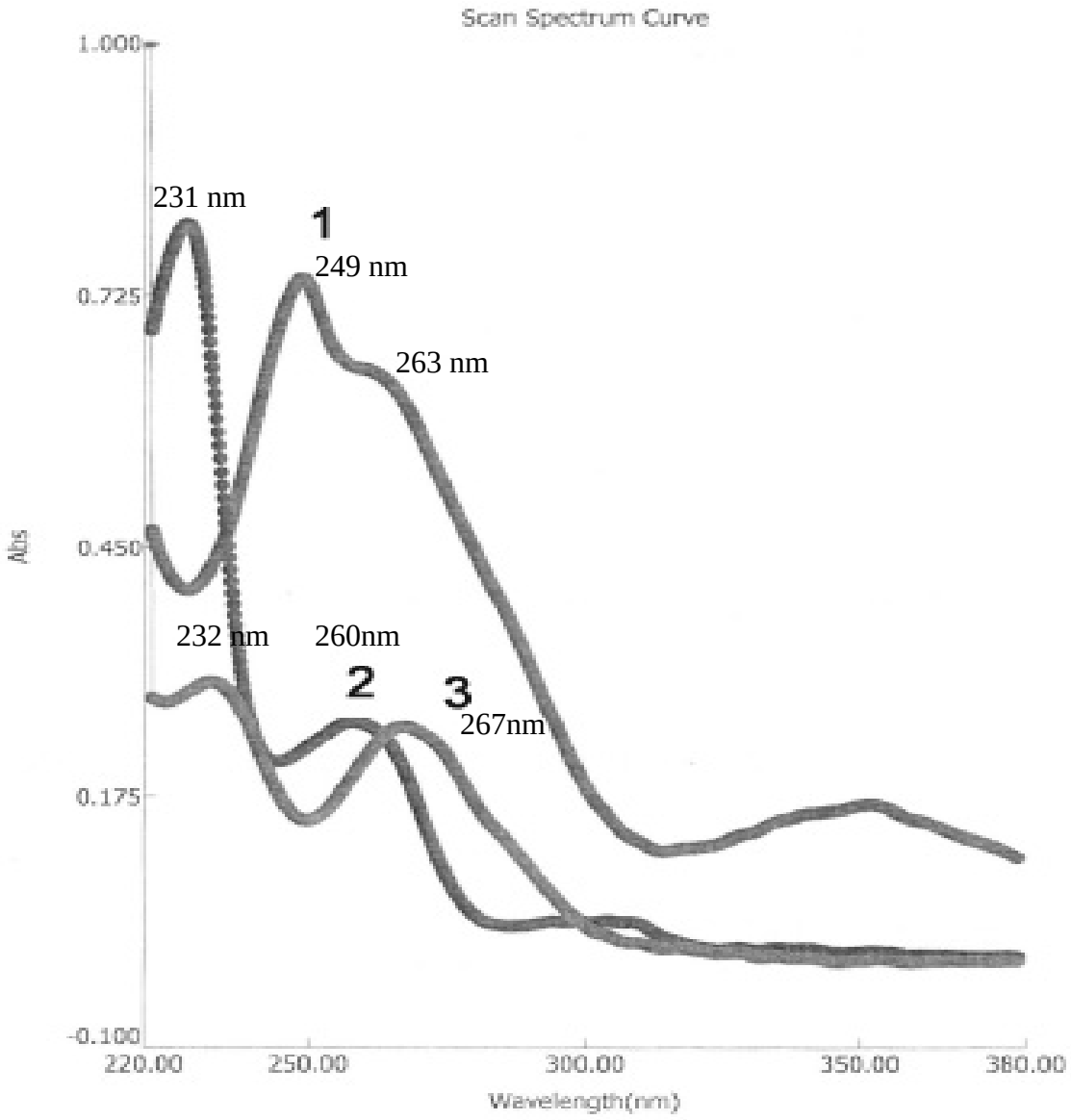


Figure (4.1): Overlaid UV-Spectra of the mixture of sulfaquinoxaline sodium (1), menadione sodium bisulfite (2) and amprolium hydrochloride (3), in the mobile phase (ACN: 0.2 M NH_4AC buffer (85:15; v/v).

First, we tried the reversed phase mode (RP- HPLC) by using different percentages of acetonitirle and 0.2 M ammonium acetate adjusted to pH 5.7 with glacial acetic acid as a mobile phase. The column was C_{18} (5µm, 250mm × 4.6mm). Upon testing all the mobile phases, no promising separation was achieved (i.e. all of them get eluted with the void peak) even when the concentration of ACN was minute (0.5%). It was expected that separation by RP-HPLC was impossible for such very polar compounds.

Then, as an alternative, we tried ion pair chromatography in which the mobile phase was a mixture prepared by dissolving 2.0 g of sodium 1-hexanesulfonate in 500 ml of water; 12

ml of glacial acetic acid, 2.0 ml of triethylamine, 450 ml of methanol, and 50 ml of acetonitrile. The column used was C_{18} (5µm, 250mm × 4.6mm). Poor separation profile was obtained (as shown in figure 4.2) where all the peaks were close to each other with no baseline separation and the retentions of all compounds were almost close to the void peak.

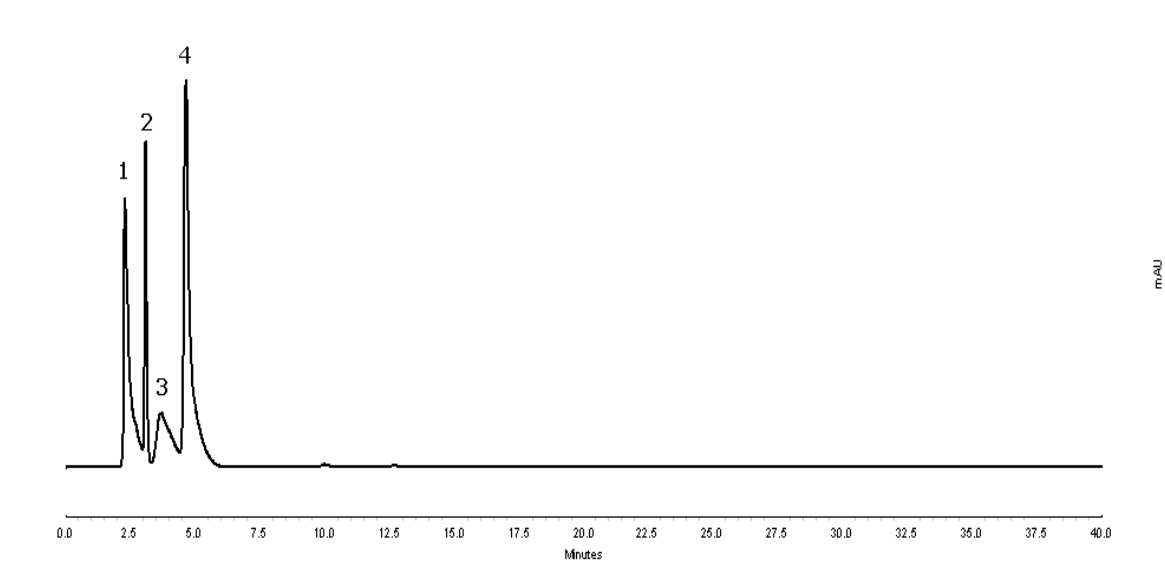


Figure (4.2): A typical chromatogram of mixture of sulfaquinoxaline sodium (4), sulfaquinoxaline related compound A (3), vitamin K_3 (1) and amprolium hydrochloride (2); Column, C_{18} (5µm, 250mm × 4.6mm) ; Mobile phase consisting of 2.0 g of sodium 1-hexanesulfonate in 500 ml of water; 12 ml of glacial acetic acid, 2.0 ml of triethylamine, 450 ml of methanol and 50 ml of acetonitrile; flow rate 1.0 ml/min; λ =274nm; column temperature=25°C; Autosampler Temp.= 15°C.

Another mobile phase was a mixture of 400 ml methanol, 600 ml water containing 1.0 g of sodium 1-decanesulfonate, 5 ml triethylamine, and 25 ml glacial acetic acid. The column used was C_8 (5µm, 250mm × 4.6mm). Broad peak profiles were produced for all the compounds with high tailing factor (Figure 4.3). Moreover, the sensitivity was decreased to more than 40% for all peaks when compared to same mixture concentration analyzed by HILIC mode.

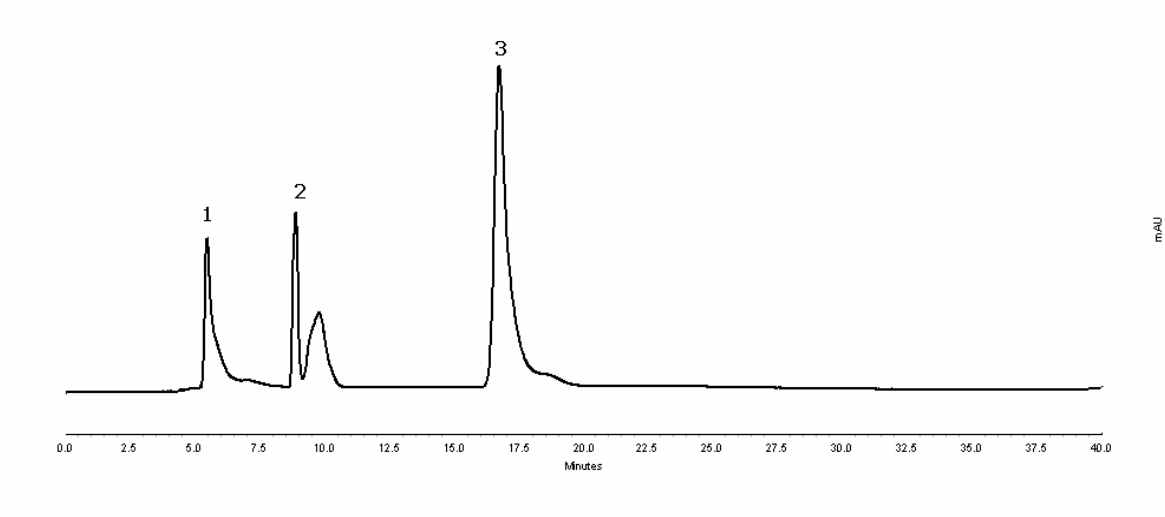


Figure (4.3): A typical chromatogram of mixture of sulfaquinoxaline sodium (3), vitamin K_3 (1) and amprolium hydrochloride (2); Column, C_8 (5μm, 250mm × 4.6mm); Mobile phase consisting of a mixture of 400 ml methanol, 600 ml water containing 1.0 g of sodium 1-decanesulfonate, 5 ml triethylamine, and 25 ml glacial acetic acid; flow rate 1.0 ml/min; λ =274nm; column temperature=25°C; Autosampler Temp. = 15°C.

It is clear that the use of ion pair reagents decreased the assay sensitivity for all compounds particularly vitamin K_3 since it is present at 0.2% level of the concentration of other compounds in the mixture. Therefore, increasing the concentration of the ion pair reagent will greatly lower the sensitivity of vitamin K_3 and increase the cost of analysis due to the expensive cost of ion-pair reagents. The unsatisfactory results obtained by RP and PIC, aided us in switching to HILIC technology. Utilizing this column spare the use of ion-pair reagents as well as naturally increasing the sensitivity of the eluted peaks and reducing the total analysis cost.

The ZIC®-HILIC column (25.0 cm long ×4.6 mm i.d., dp=5 μm) was the next choice because of its suitability for the separation of a wide range of very polar and hydrophilic compounds. In addition, the separation of ionic and very polar compounds could be achieved by using this column with polar mobile phase that contains high portions of organic solvent.

During the method development phase of this research work, different compositions of the mobile phase have been employed. These mobile phases differ in the pH, concentration of

NH_4AC buffer, and organic solvent percentages. Moreover, different temperatures for the chromatographic column have been tried.

Our first choice of a mobile phase was ACN/10 mM NH_4AC buffer solution (80:20; v/v). The pH of the mobile phase was adjusted to pH of 5.0. Typically, as recommended by the column manufacturer, the mobile phase for HILIC consists of 40-97% ACN in water. To obtain reproducible results at least 3% of water should be maintained in the mobile phase. This amount of water is necessary to ensure sufficient hydration of the stationary phase particles. To the contrary of reversed phase, the ACN, one of the weaker solvents in HILIC, provides a much higher increase in retention compared to other solvents like methanol. Suitable buffer for HILIC is ammonium salt of acetate due to its excellent solubility even in very high concentrations of organic solvent. It was found that no baseline separation was achieved for our active ingredients, i.e.; sulfaquinoxaline sodium and vitamin K_3. Even amporlium hydrochloride was separated with total analysis time close to 40 minutes, as shown in figure 4.4 and Table 4.1.

4.1.1. The effect of buffer concentration:

When the concentration of NH_4AC was doubled (i.e., 20 mM) overlapped peaks with no baseline separation was achieved between sulfaquinoxaline sodium and vitamin K_3 compounds. The amprolium hydrochloride eluted far a way with a total analysis time of about 30 minutes, as shown in figure 4.4 and Table 4.1.

When the ionic strength in the mobile phase was increased to 30, 40, and 50 mM NH_4AC respectively, a baseline separation emerged with more reasonable resolution values **(Rs).** At this point, it was anticipated that the separation might be better with the increase of the concentration of ammonium acetate.

Upon increasing NH_4AC concentration to 75mM, clear separated peaks evolved. However, a new tiny peak between sulfaquinoxaline sodium and vitamin K_3 emerged which is due to sulfaquinoxaline impurity (appears to be a degradation product) as seen in figure 4.5. In order to improve the resolution, the concentration of NH_4AC was further increased.

Therefore, solutions of 0.1 and 0.2 M NH_4AC were tried. A much more pronounced baseline separation were obtained with **Rs** of 2.71 between sulfaquinoxaline sodium and its impurity and **Rs** of 2.1 between sulfaquinoxaline sodium impurity and vitamin K_3 at 0.1M. The tailing factor of amproulim hydrochloride was 2.51. In the case of 0.2 M, the **Rs** of 2.3 between sulfaquinoxaline sodium and its impurity and Rs of 5.74 between sulfaquinoxaline sodium impurity and vitamin K_3 was seen.

The best separation and baseline resolution was achieved by using 0.2 M concentration with **Rs >2.0** and total analysis time was less than 16 minutes (Figure 4.4, 4.6, table 4.1).

The retention was slightly decreased by increasing ionic strength (salt concentration). This seems to imply that retention time was mainly based on hydrophilic interaction as the ionic strength increased, the positively charged ammonium ions in the mobile phase may weaken the hydrophilic interaction between protonated compounds and stationary phase which result in shortening the retention time

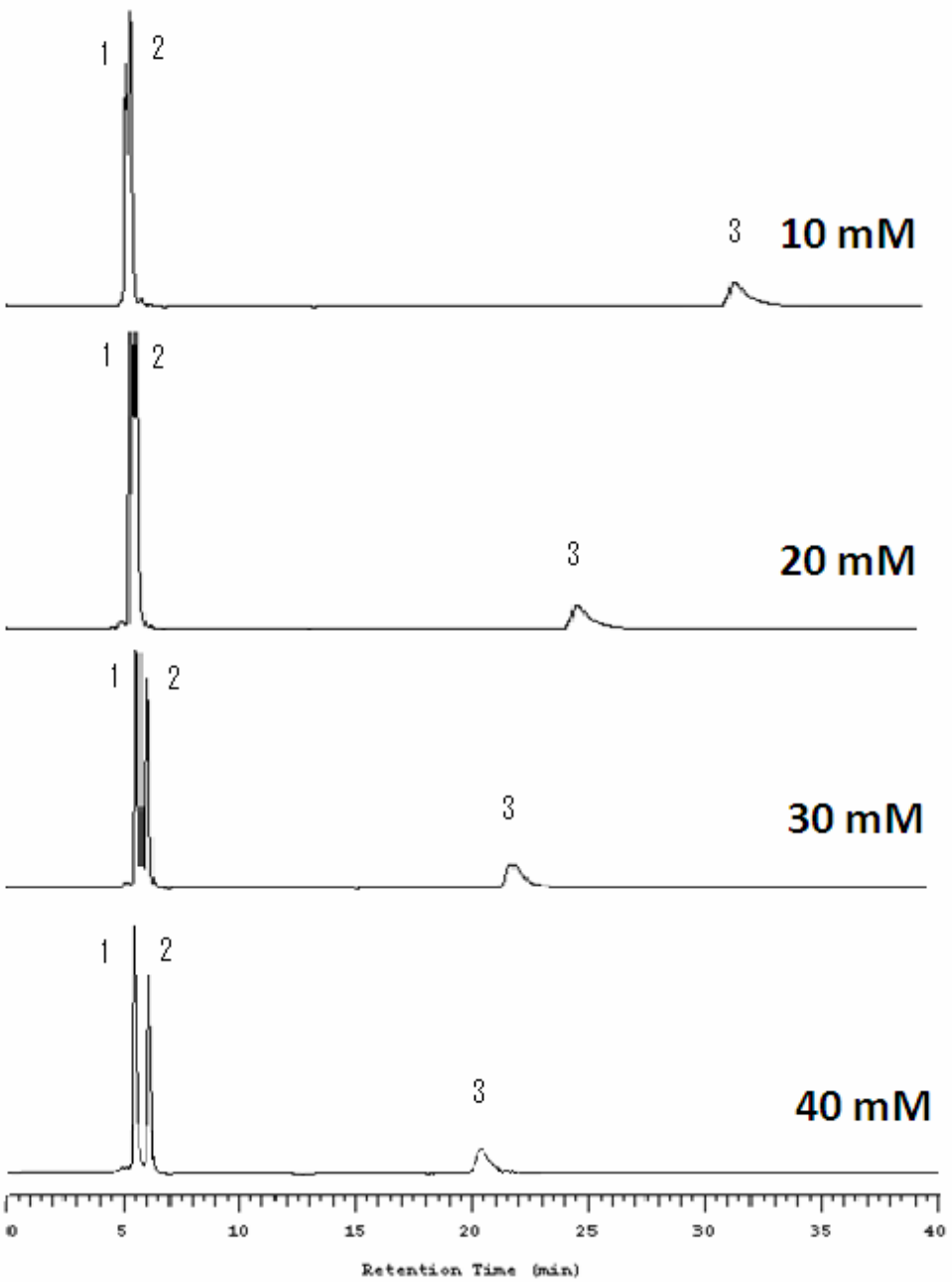


Figure (4.4): Chromatograms obtained upon 20µl injection of a mixture of sulfaquinoxaline sodium (1) (20µg/ml), Vitamin K_3 (2) (10µg/ml), and amprolium hydrochloride (3) (20µg/ml). Mobile phase consisting of ACN and NH_4AC solution (80:20; v/v) adjusted to pH of 5.0; flow rate =0.5 ml/min; λ =263nm; the concentration of NH_4AC is as shown in the figure.

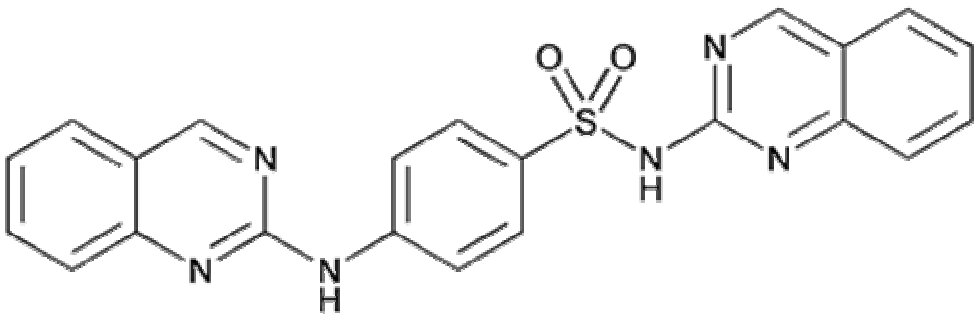


Figure (4.5): Structure of Sulfaquinoxaline Related Compound A. [55]

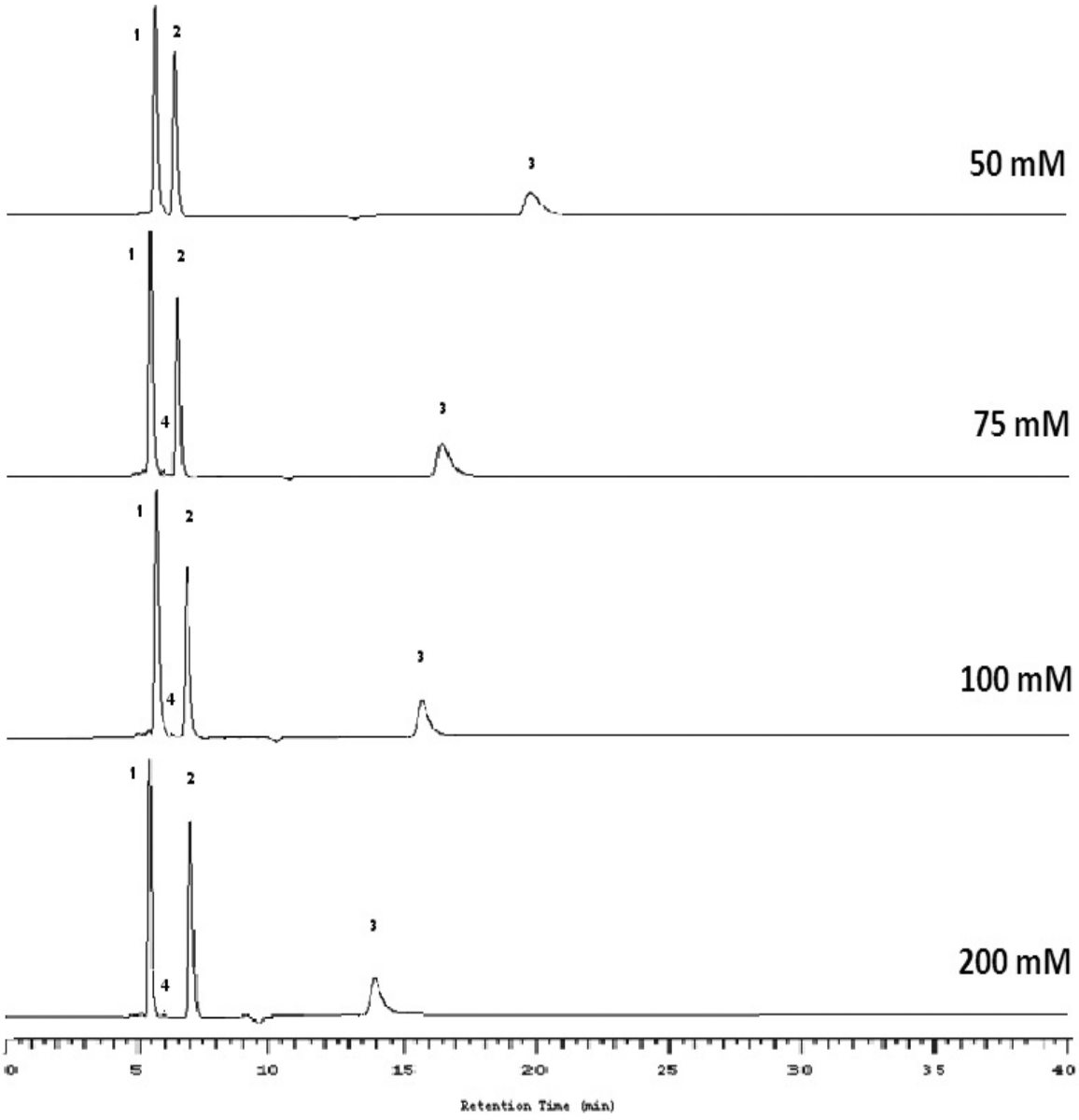


Figure (4.6): Chromatograms obtained upon 20µl injection of a mixture of sulfaquinoxaline sodium (20µg/ml) (1), Vitamin K_3 (2) (10µg/ml), and amprolium hydrochloride (3) (20µg/ml), sulfaquinoxaline impurity (4). Mobile phase consisting of ACN and NH_4AC solution (80:20; v/v) adjusted to pH of 5.0; flow rate =0.5 ml/min; λ =263nm; the concentration of NH_4AC is as shown in the figure.

Table (4.1): Effect of concentration of ammonium acetate on resolution (Rs)

Concentration of NH_4AC solution (M)	*Rs $_{1.4}$	†Rs $_{2.4}$	‡Rs$_{1.2}$	ζRs $_{2.3}$
0.01	0	0	0.89	25.51
0.02	0	0	0.74	22.92
0.03	0	0	1.47	21.7
0.04	0	0	2.38	20.86
0.05	0	0	2.87	20.27
0.075	2.78	1.38	4.1	17.42
0.1	2.71	2.1	4.8	16.16
0.2	2.3	3.4	5.74	13.54

***Rs $_{1.4}$:** Represents the resolution value between sulfaquinoxaline sodium (1) and its impurity (4).

†Rs $_{2.4}$: Represents the resolution value between vitamin K_3 (2) and sulfaquinoxaline sodium Impurity (4)

‡Rs$_{1.2}$: Represents the resolution value between sulfaquinoxaline sodium (1) and vitamin K_3 (2).

ζ Rs $_{2.3}$: Represents the resolution value between vitamin K_3 (2) and amprolium HCl (3)

Resolution (Rs): The ratio of the distance between two peak maxima to the mean value of the peak width at the base line [56]

$$Rs = 2(TR2 - TR1) / (W1 + W2)$$

$T_{R2:}$ Retention time of the second eluting peak

$T_{R1:}$ Retention time of the first eluting peak

$W_{1:}$ Peak width of the first peak at the base line

$W_{2:}$ Peak width of the second peak at the base line

4.1.2. The effect of pH:

A study of pH effects on the resolution of A.S.K compounds was deemed necessary to further optimize the separation conditions. The resolution values for sulfaquinoxaline sodium, sulfaquinoxaline sodium impurity, vitamin K_3, and amprolium hydrochloride were significantly influenced by the variation of the pH of the mobile phase.

We tried different pH values such as 3.5, 4.0, 4.5, 5.7, 6.0, 6.5 and 7.0. As shown in figure 4.7 and table 4.2, variation of pH yielded maximum resolution for all compounds at pH 5.7. In case of pH$\leq$ 4.0, interference occurred between sulfaquinoxaline sodium and its impurity. The **Rs** between vitamin K_3 and amprolium hydrochloride was decreased to 1.47 at pH of 4.5 even there is good **Rs** between sulfaquinoxaline sodium and sulfaquinoxaline sodium impurity. At pH of 6.0, baseline separation was achieved between sulfaquinoxaline sodium and its impurity and between sulfaquinoxaline sodium impurity and vitamin K_3 but the **Rs** values were 1.8 and 1.7, respectively.

At pH of 5.7, excellent separation was achieved between all the combined drugs as well as the impurity. When the pH was raised by 0.3 and 0.8 increments namely at 6.0 and 6.5 respectively, the **Rs** $_{1.4}$ were 1.78 and 1.7, respectively and **Rs** $_{2.4}$ were 1.7 and 1.6, respectively. At pH of 7.0, complete interference occurred between sulfaquinoxaline sodium and its related impurity.

Therefore, pH 5.7 was selected as optimum value to which a maximum **Rs** was achieved for all the combined drugs as shown in figure 4.7 and table 4.2.

This behavior may happen because when the mobile phase pH increased significantly, more surface silanol groups of the stationary phase were deprotonated ($-Si-O^{-}$). The electrostatic interactions between the positively charged basic compounds and negatively charged surface silanol groups increased resulting in increase of retention time of compounds.

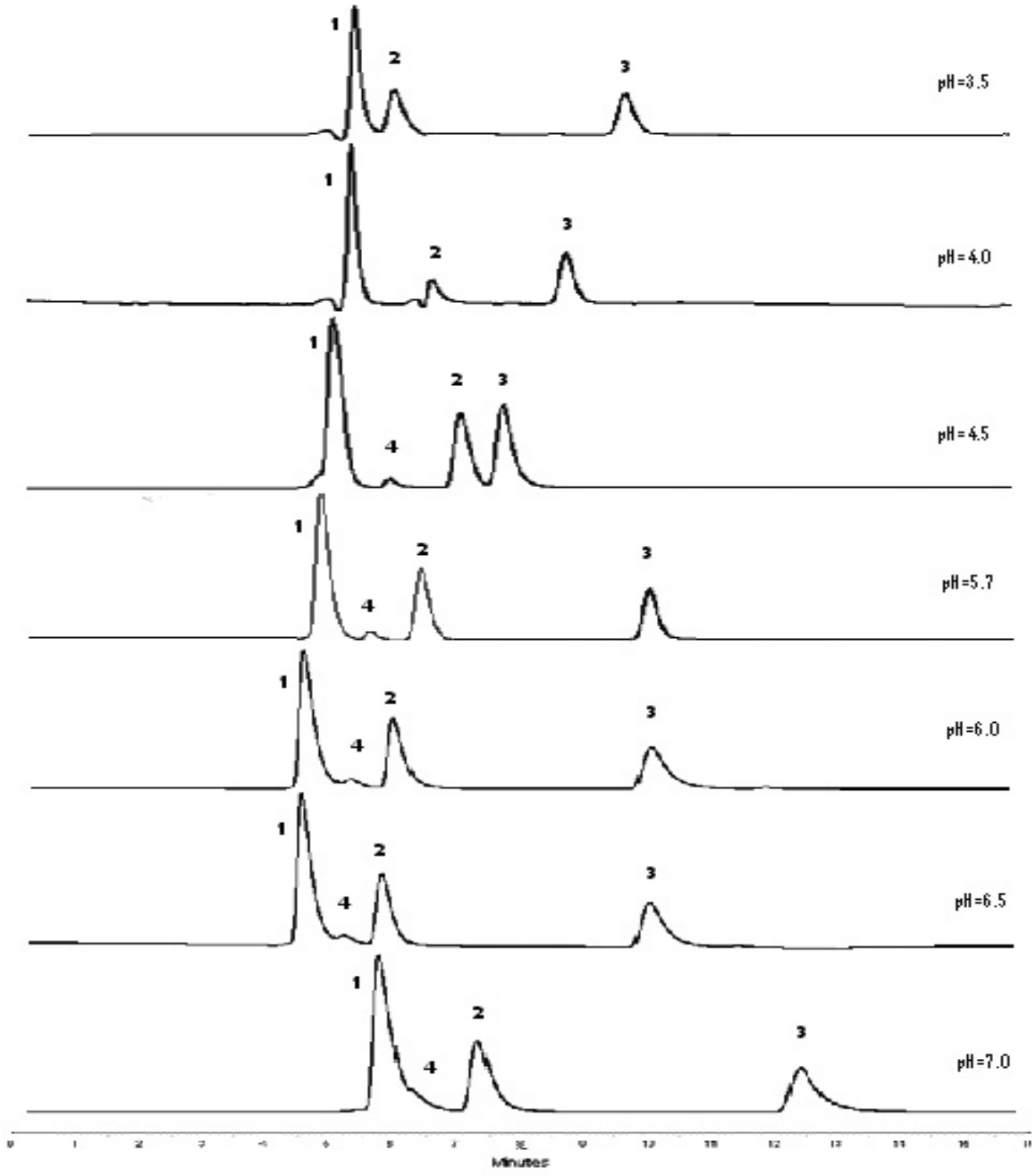


Figure (4.7): Chromatograms obtained upon 20µl injection of a mixture of sulfaquinoxaline sodium (1), Vitamin K_3 (2), and amprolium hydrochloride (3). Mobile phase consisting of ACN and NH_4AC solution (80:20; v/v), at different pH's; flow rate 0.5 ml/min; λ =263nm. Note: peak (4) is due to sulfaquinoxaline sodium impurity.

Table (4.2): Effect of pH on resolution (Rs)

pH	*Rs $_{1.4}$	†Rs $_{2.4}$	‡Rs$_{1.2}$	ζRs $_{2.3}$
3.5	0	0	1.74	7.89
4.0	0	0	4.35	5.6
4.5	2.3	2.8	4.5	1.47
5.7	2.14	2.2	3.9	7.3
6.0	1.78	1.72	3.4	7.2
6.5	1.7	1.6	3.3	7.6
7.0	0	0	2.7	7.2

***Rs $_{1.4}$:** Represents the resolution value between sulfaquinoxaline sodium (1) and its impurity (4).

†Rs $_{2.4}$: Represents the resolution value between vitamin K_3 (2) and sulfaquinoxaline sodium Impurity (4)

‡Rs$_{1.2}$: Represents the resolution value between sulfaquinoxaline sodium (1) and vitamin K_3 (2).

ζ Rs $_{2.3}$: Represents the resolution value between vitamin K_3 (2) and amprolium HCl (3)

4.1.3. The effect of acetonitrile percentage:

The effect of acetonitrile organic solvent percentage on **Rs** was also investigated. The ACN percentages combined with 0.2 M NH_4AC aqueous buffer were 60, 70, 80, 85, 90, and 95% ACN. It was found that increasing the ACN ratio increased the retention time and improved the **Rs.** The best **Rs** value was achieved by using 85%as shown in figure 4.8 and table 4.3.

When the ACN was 60%, interference occurred between sulfaquinoxaline sodium and its impurity and the **Rs** were decreased for all compounds. Analysis time was about 11 minutes. At this point, it was anticipated that increasing the ACN concentration could improve the baseline separation and increase the **Rs** values between all compounds. Therefore, a systematic increase was tried with increment of 10% and 5%. When a 70%

ACN was tested, a better baseline separation achieved but still not satisfactory, when the ACN increased to 80%, a better separation was achieved. In all cases the pH was fixed to 5.7.

When 85% ACN was used at pH of 5.7, a separable four peaks was seen with a better resolution. At 90% ACN, the chromatogram reveals the best **Rs** values between sulfaquinoxaline sodium and its impurity with a total analysis time of about 23 minutes. The main drawback was the high value of analysis time which means it is time consuming. Finally, upon switching to 95% the last eluting peaks merged together as shown in the figure. Table 4.3 and figure 4.8 shows that the optimized mobile phase prepared from that system for the separation of all compounds was 0.2 M NH_4AC: ACN (15:85; v/v), at pH of 5.7.

Figure 4.8 illustrates the change of retention time with varying ratios of ACN from 60-95%. The results show that the content of ACN in the mobile phase has great influence on the resolution and selectivity. By increasing the %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 compounds. Our results indicated that the retention time was primarily governed by typical hydrophilic and electrostatic interactions between the solutes and the stationary phase.

The effect of acetonitrile on retention was systematically behaved up to 90% ACN level. However, when an extra 5% ACN was added, the peak of vitamin K_3 was eluted in close proximity to amprolium HCl. This probably indicates a sudden change in equilibrium of this peak in the HILIC system.

Table (4.3): Effect of acetonitrile percentage on the resolution values of compounds separated

% ACN	*Rs $_{1.4}$	†Rs $_{2.4}$	‡Rs$_{1.2}$	ζRs $_{2.3}$
60	0	0	2.04	3.7
70	2.2	1.0	2.6	4.7
80	2.1	2.1	3.5	7.15
85	2.2	3.3	5.0	10.2
90	2.5	6.2	7.8	8.8
95	3.97	21.1	21.5	1.05

***Rs $_{1.4}$:** Represents the resolution value between sulfaquinoxaline sodium (1) and its impurity (4).

†Rs $_{2.4}$: Represents the resolution value between vitamin K_3 (2) and sulfaquinoxaline sodium Impurity (4)

‡Rs$_{1.2}$: Represents the resolution value between sulfaquinoxaline sodium (1) and vitamin K_3 (2).

ζ Rs $_{2.3}$: Represents the resolution value between vitamin K_3 (2) and amprolium HCl (3)

It is worthwhile mentioning that during the whole work, we chose to use Rs value as the chromatographic parameter since it reflects the column efficiency and selectivity. The relation between the three parameters is as shown below:

$$R = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{1 + k'_B}{k'_B}\right)$$

N: Number of theoretical plates

α: Selectivity factor

K_B: Capacity factor for compound B

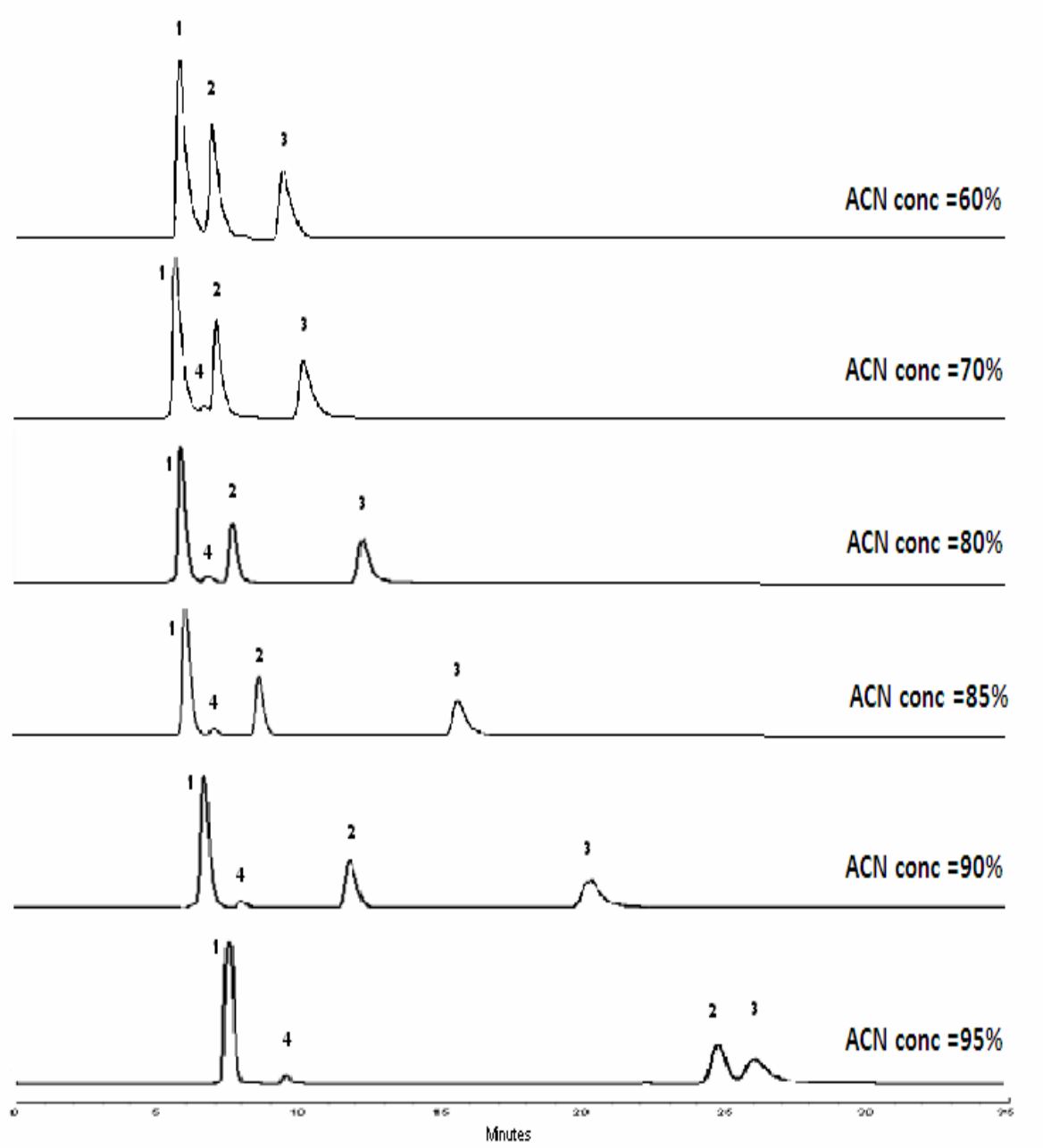


Figure (4.8): The separation obtained with 20µl injection of a mixture of sulfaquinoxaline sodium (1), Vitamin K_3 (2), and amprolium hydrochloride (3), Sulfaquinoxaline impurity (4). Mobile phase consisting of ACN and 0.2M NH_4AC solution, at pH of 5.7; flow rate 0.5 ml/min; λ =263nm. The %ACN is as shown in the figure.

4.1.4. The effect of methanol percentage:

This time, we tried to use methanol (MeOH) in addition to ACN as organic solvent modifier by using different percentages of MeOH with ACN and constant buffer concentration of 0.2 M NH_4AC (10:75:15; v/v/v) at pH of 5.7. We found that by using 10% methanol, there is no significant effect on **Rs** values of all compounds as shown in figure 4.9 and table 4.4. By increasing the % MeOH up to 20%, we observed an increase in the resolution value between sulfaquinoxaline sodium and its impurity. We also observed a decrease in the Rs values between sulfaquinoxaline sodium impurity and vitamin K_3 and between vitamin K_3 and amprolium hydrochloride. The total analysis time was about 15 minutes.

At this stage, it was anticipated that using trinary solvent mixture, namely MeOH, ACN and buffer will either have no significant effect on the **Rs** of all compounds or would decrease the **Rs** since MeOH is stronger solvent compared to ACN using HILIC system and therefore may decrease the retention time and **Rs.**

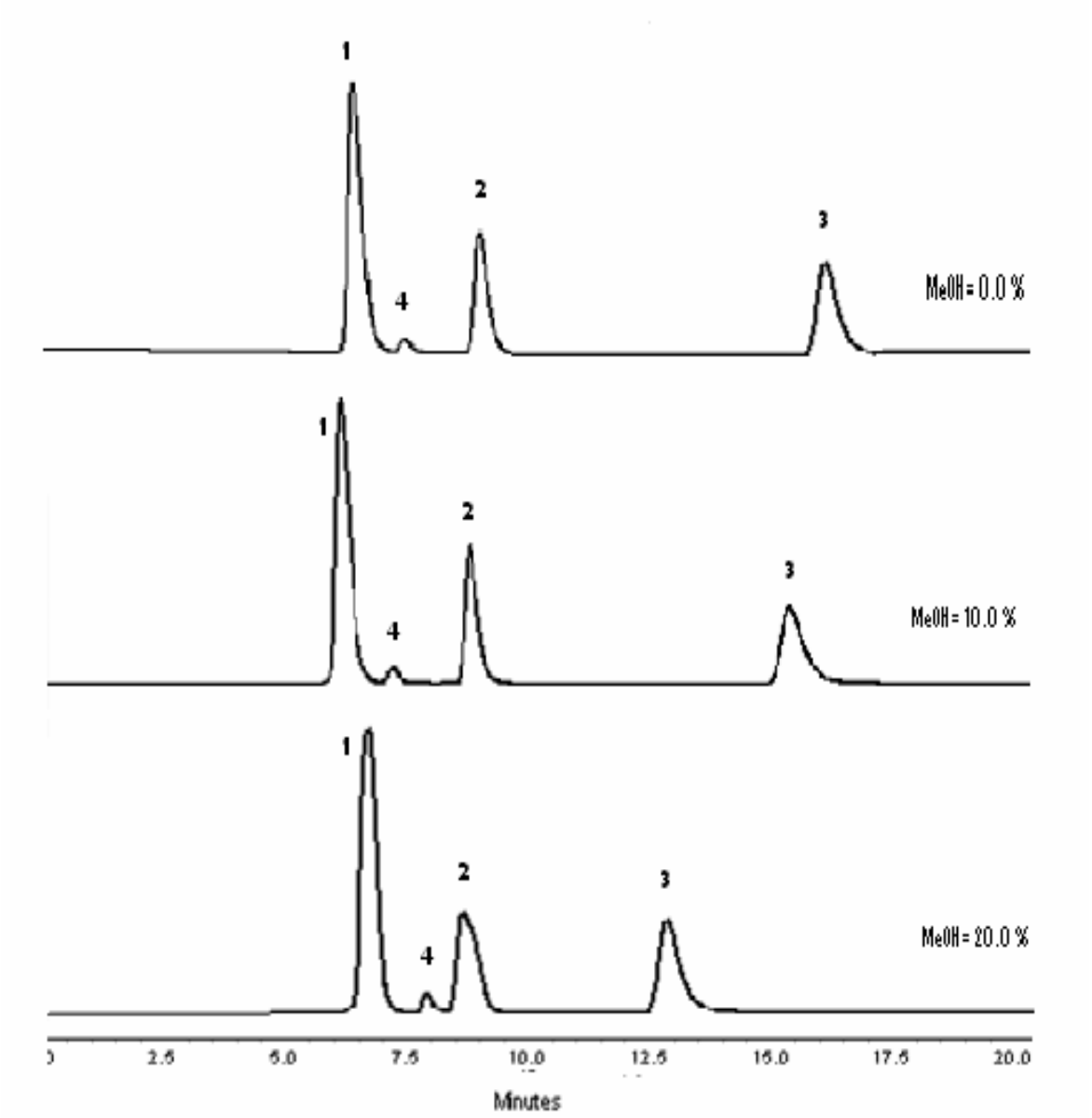


Figure (4.9): The separation obtained with 20µl injection of a mixture of sulfaquinoxaline sodium (1), Vitamin K_3 (2), and amprolium hydrochloride (3), Sulfaquinoxaline impurity (4) at mobile phase consisting of MeOH with ACN and 0.2 M NH_4AC (10 :75 :15; v/v/v) at pH of 5.7 and MeOH with ACN and 0.2 M NH_4AC (20 :65 :15; v/v/v) at pH of 5.7, respectively; flow rate 0.5 ml/min; λ =263nm.

Table (4.4): Effect of adding methanol to mobile phase on resolution values

% MeOH	*Rs $_{1.4}$	†Rs $_{2.4}$	‡Rs$_{1.2}$	ζRs $_{2.3}$
0	2.2	3.3	5.0	10.2
10	2.16	3.6	5.4	9.94
20	2.9	2.55	5.0	5.3

***Rs $_{1.4}$:** Represents the resolution value between sulfaquinoxaline sodium (1) and its impurity (4).

†Rs $_{2.4}$: Represents the resolution value between vitamin K_3 (2) and sulfaquinoxaline sodium Impurity (4)

‡Rs$_{1.2}$: Represents the resolution value between sulfaquinoxaline sodium (1) and vitamin K_3 (2).

ζ Rs $_{2.3}$: Represents the resolution value between vitamin K_3 (2) and amprolium HCl (3)

1.4.5. The effect of temperature:

The effect of the column temperature on the resolution and tailing factor was another chromatographic parameter that was studied during optimization of HPLC methods. Different temperatures such as 15°C, 20°C, 25°C, 30°C, were evaluated. It was clear that varying temperatures between 15 °C and 30 °C have very little influence on Rs values as shown in figure 4.10 and table 4.5.

The effect of temperature on the tailing factor was also determined. The best tailing factor obtained was at temperature of about 25°C and 30°C.

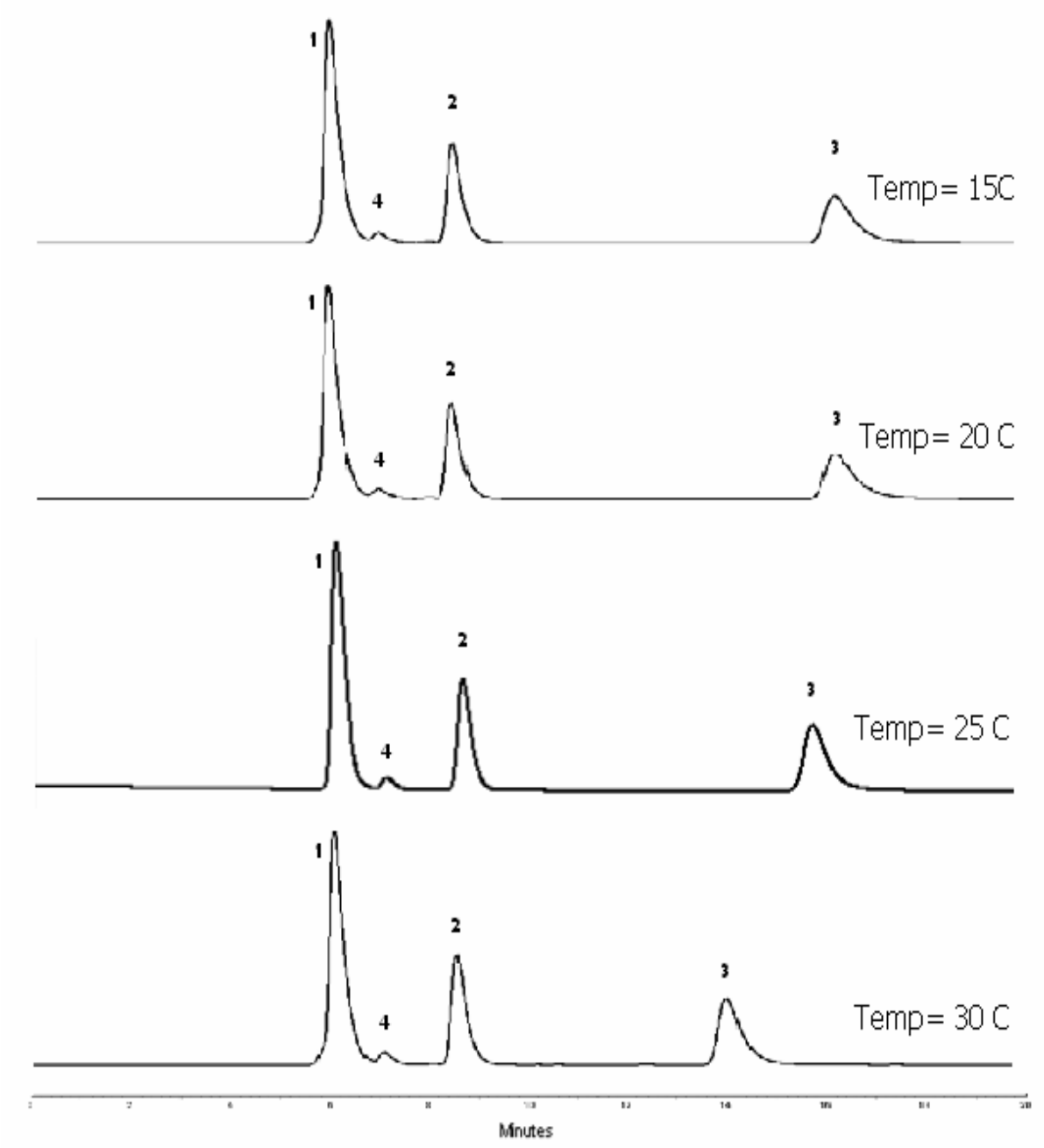


Figure (4.10): Chromatograms obtained upon 20µl injection of a mixture of sulfaquinoxaline sodium (1), Vitamin K_3 (2), and amprolium hydrochloride (3), Sulfaquinoxaline impurity (4) at mobile phase consisting of ACN and 0.2M NH_4AC solution (85:15; v/v) at pH 5.7; flow rate 0.5 ml/min; λ =263nm. The column temperature is as shown in the figure.

Table (4.5): Effect of column temperature on resolution values

Temperature (°C)	*Rs $_{1.4}$	†Rs $_{2.4}$	‡Rs$_{1.2}$	ζRs $_{2.3}$
15°C	2.0	2.9	4.7	9.6
20°C	2.07	3.0	4.6	9.2
25°C	2.2	2.6	4.3	10.1
30°C	2.14	3.1	4.7	8.0

***Rs $_{1.4}$:** Represents the resolution value between sulfaquinoxaline sodium (1) and its impurity (4).

†Rs $_{2.4}$: Represents the resolution value between vitamin K_3 (2) and sulfaquinoxaline sodium Impurity (4)

‡Rs$_{1.2}$: Represents the resolution value between sulfaquinoxaline sodium (1) and vitamin K_3 (2).

ζ Rs $_{2.3}$: Represents the resolution value between vitamin K_3 (2) and amprolium HCl (3)

Table (4.6): Effect of column temperature on tailing factor

Temperature (°C)	*T_F(1)	† T_F (2)	‡T_F (3)
15°C	1.4	1.35	1.67
20°C	1.35	1.33	1.54
25°C	1.3	1.31	1.45
30°C	1.3	1.29	1.48

***T_F (1):** Represents the tailing factor of sulfaquinoxaline sodium (1)

† T_F (2): Represents the tailing factor of vitamin K_3 (2)

‡T_F(3): Represents the tailing factor of amprolium HCl

Tailing factor (T_F): The tailing factor of a peak is calculated from the expression: [57]

$$T_F = \frac{W\ 0.05}{2d}$$

$\mathbf{w_{0.05}}$ = Width of the peak at one-twentieth of the peak height,

d = Distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

As shown from all the accumulated results, the optimized mobile phase consisted of a mixture of 0.2M NH_4AC solution and ACN organic solvent (15:85; v/v); at pH 5.7 at room temperature. During the method development process, the concentration of the compounds prepared was about 20µg/ml of sulfaquinoxaline sodium, about 10µg/ml of Vitamin K_3, and about 20µg/ml of amprolium hydrochloride. Figure 4.11 shows typical chromatograms obtained for individual components and for the mixture simultaneously using the optimized conditions.

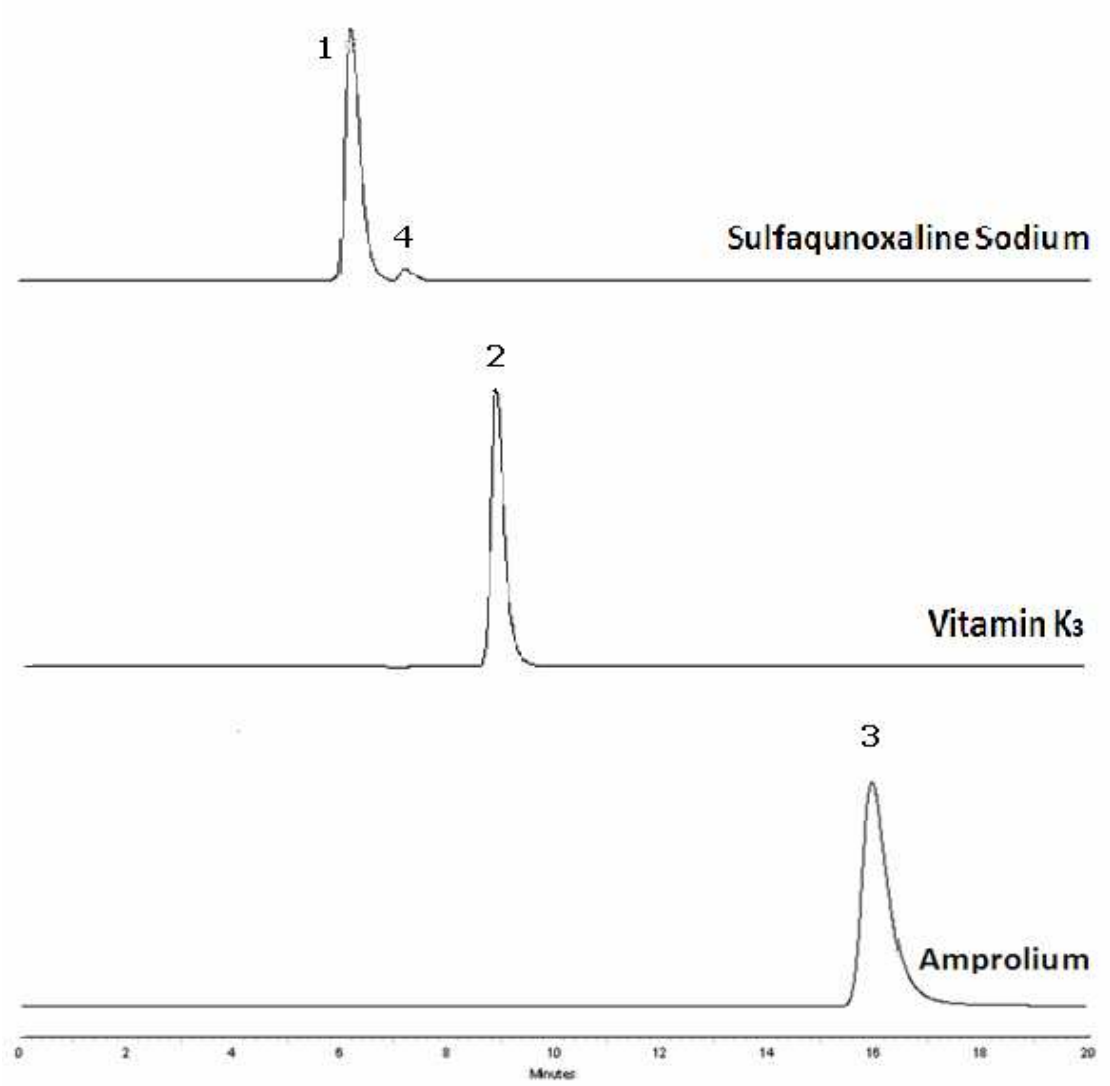


Figure (4.11): Typical chromatograms obtained upon 20 µl separate injections of sulfaquinoxaline sodium, vitamin K_3 and amprolium hydrochloride. Column, a ZIC-HILIC column (25.0 cm long ×4.6 mm i.d.) with particle size of 5 µm protected with a ZIC-HILIC guard column (20mm× 2.1mm, 5µm); Mobile phase consisting of ACN and 0.2M NH_4AC solution (85:15; v/v) at pH 5.7; flow rate 0.5 ml/min; λ =263nm; column temperature=25°C; Autosampler Temp. = 15°C.

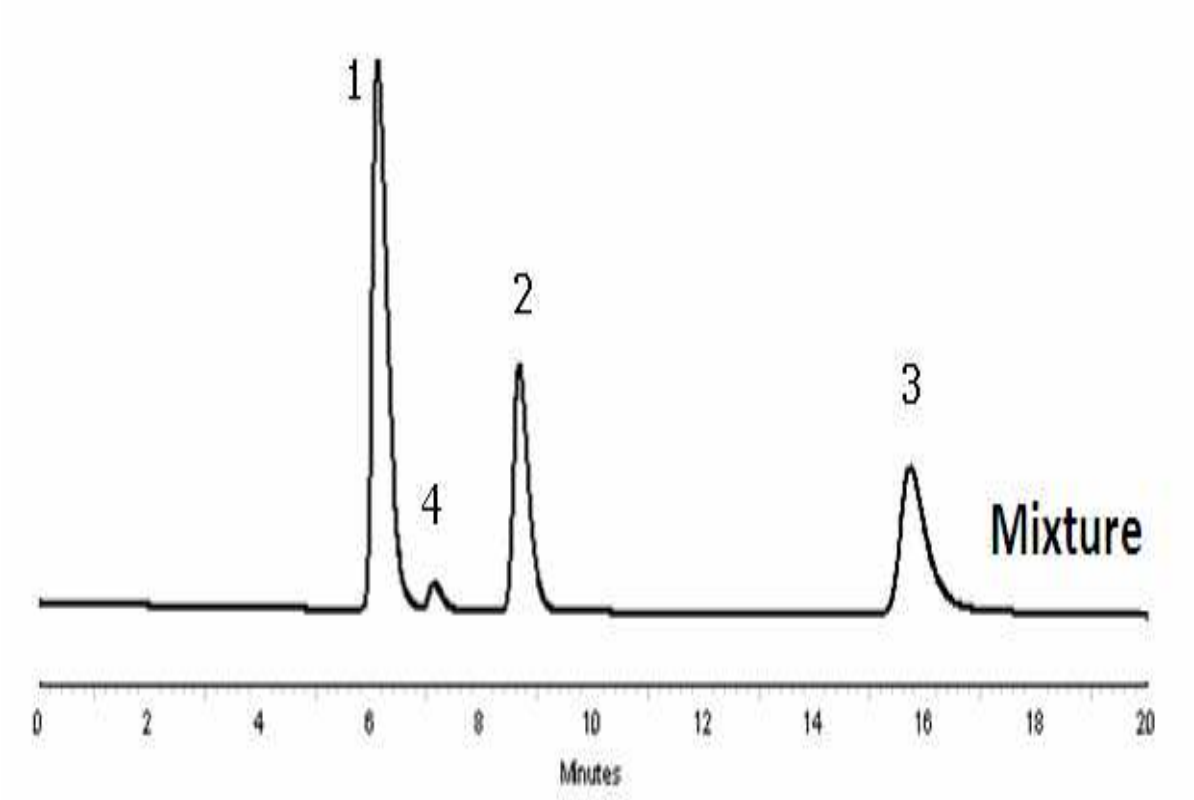
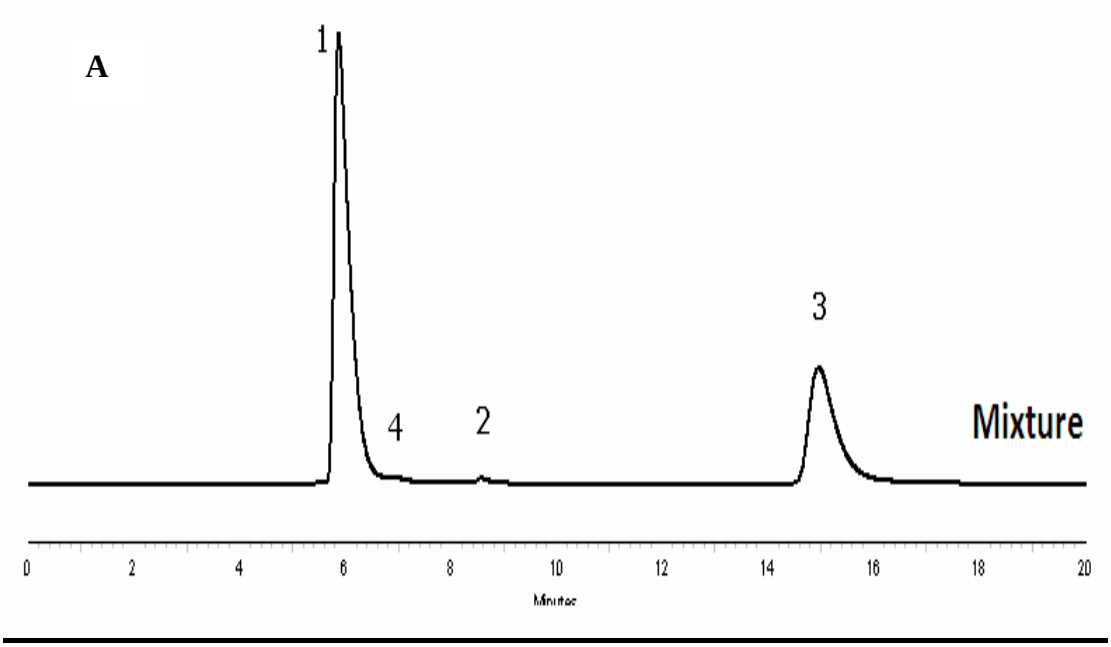


Figure (4.12): A typical chromatogram of mixture of sulfaquinoxaline sodium (1), vitamin K_3 (2) and amprolium hydrochloride (3); Column, a ZIC-HILIC column (25.0 cm long ×4.6 mm i.d.) with particle size of 5 μm protected with a ZIC-HILIC guard column (20mm× 2.1mm, 5μm); Mobile phase consisting of ACN and 0.2M NH_4AC solution (85:15; v/v) at pH 5.7; flow rate 0.5 ml/min; λ =263nm; column temperature=25°C; Autosampler Temp. = 15°C. Note: Peak (4) is due to sulfaquinoxaline impurity.

The concentration of compounds was monitored at 20μg/ml level for sulfaquinoxaline sodium, 0.2μg/ml for Vitamin K_3, and 20μg/ml for amprolium hydrochloride to resemble the concentration ratio in the A.S.K product powder, Figure 4.13 shows typical chromatogram obtained for the mixture of these three compounds simultaneously using the optimized conditions.



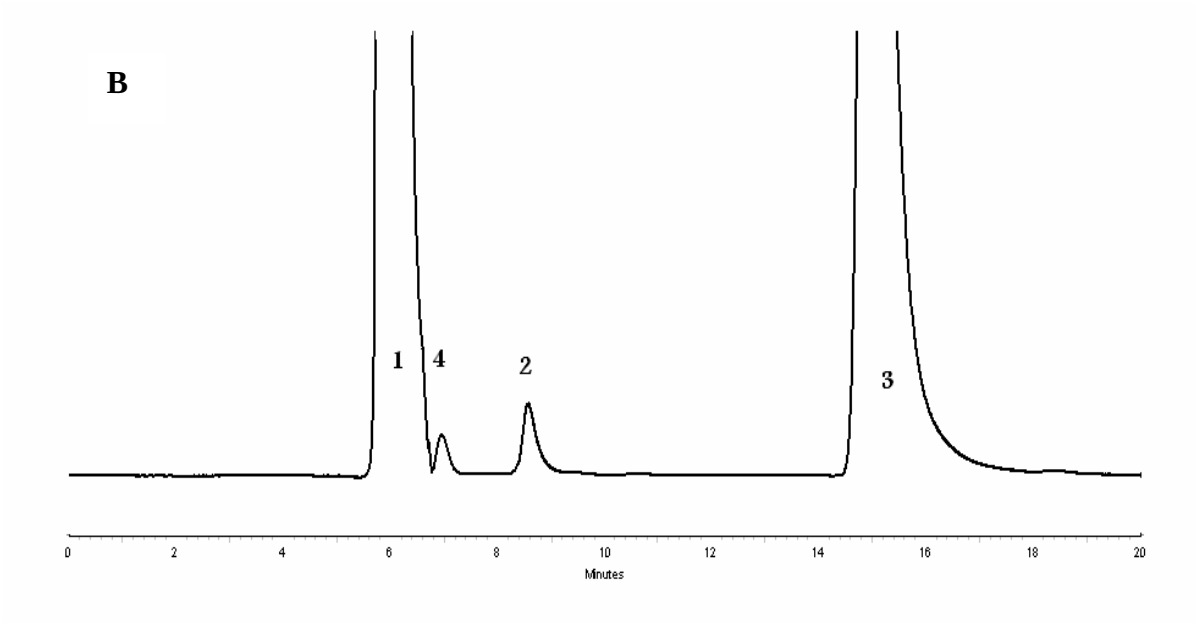


Figure (4.13): (A) typical chromatogram of mixture of sulfaquinoxaline sodium (1) (20µg/ml), vitamin K_3 (2) (0.20µg/ml) and amprolium hydrochloride (3) (20µg/ml), Peak (4) is due to sulfaquinoxaline impurity. All other chromatographic conditions are same as in figure 4.12; (B) is a zoomed view of chromatogram A.

Chapter Five

Analytical Validation of the Optimized HPLC Method

The validation was carried out according to the ICH/USP guidelines and regulations. This chapter comprises the determination of the following validation parameters: Specificity; Linearity; Range; Accuracy (recovery); Precision (repeatability and intermediate precision); Robustness and Stress test.

5.1 Specificity (placebo interference)

The chromatograms of the placebo solution, standard solution and test solution were recorded at the same wavelength in order to check the specificity of the method, (See Figure 5.1).

Assessment:

No peaks were observed when the placebo (dextrose monohydrate) that lacks chromophore was injected.

The retention time of the sulfaquinoxaline sodium, amprolium hydrochloride, and menadione (as sodium bisulfite) sample peaks match exactly to that of the appropriate peaks of the standard solution. No peaks are present at these retention times in the placebo chromatogram. Therefore, this method is suitable for the identification and the assay of

sulfaquinoxaline sodium, amprolium hydrochloride, and menadione (as sodium bisulfite) in A.S.K powder.

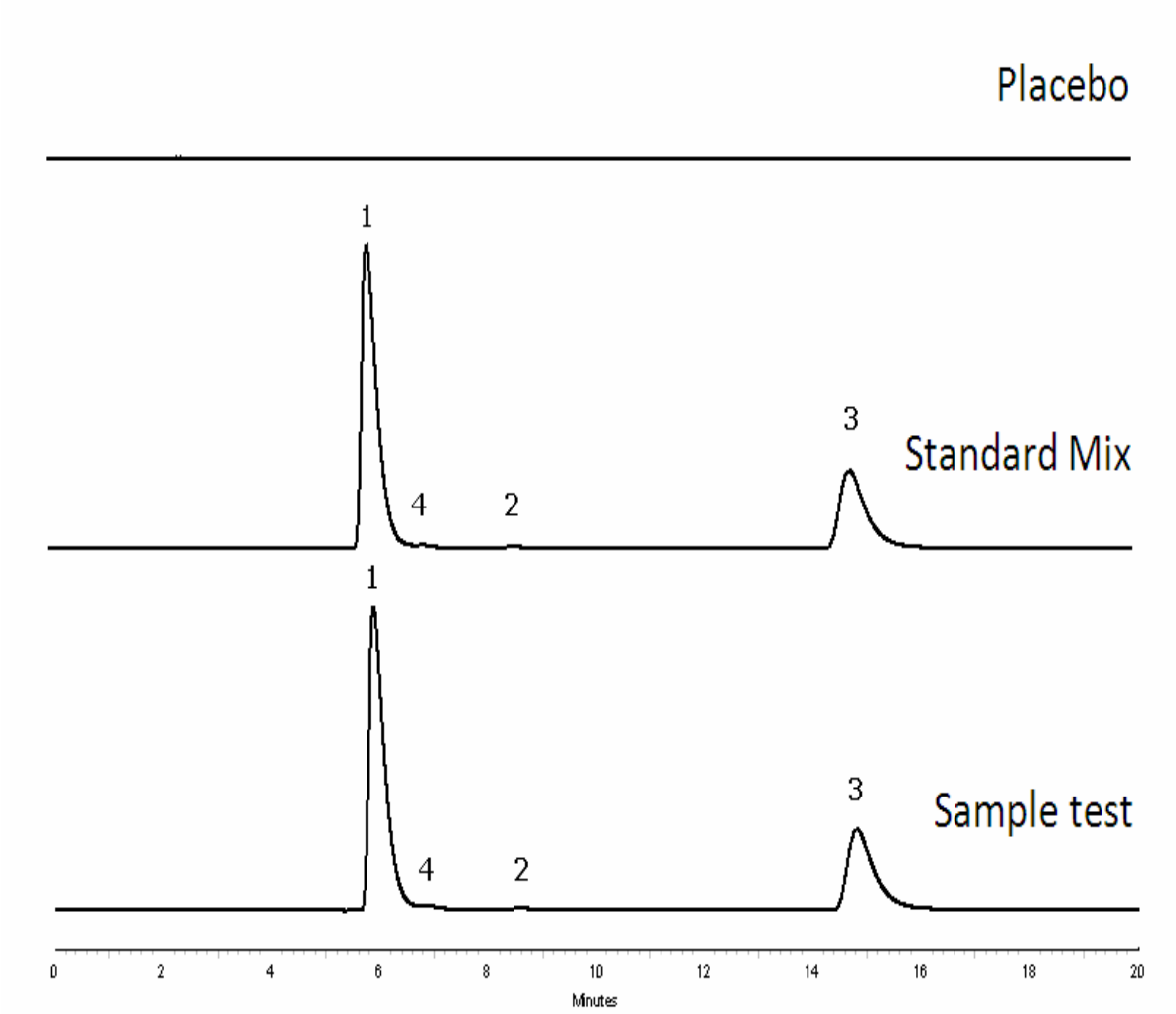


Figure (5.1): A typical chromatograms of Placebo, standard solution of sulfaquinoxaline sodium, vitamin K_3 and amprolium hydrochloride and test solution of A.S.K powder ; Column, a ZIC-HILIC column (25.0 cm long ×4.6 mm i.d.) with particle size of 5 µm protected with a ZIC-HILIC guard column (20mm× 2.1mm, 5µm); Mobile phase, acetonitrile and 0.2M ammonium acetate solution (15:85; v/v) adjusted to pH 5.7; flow rate 0.5 ml/min; λ =263nm; column temperature=25°C; Autosampler Temp. = 15°C; concentration of vitamin K_3 was about 0.2µg/ml.

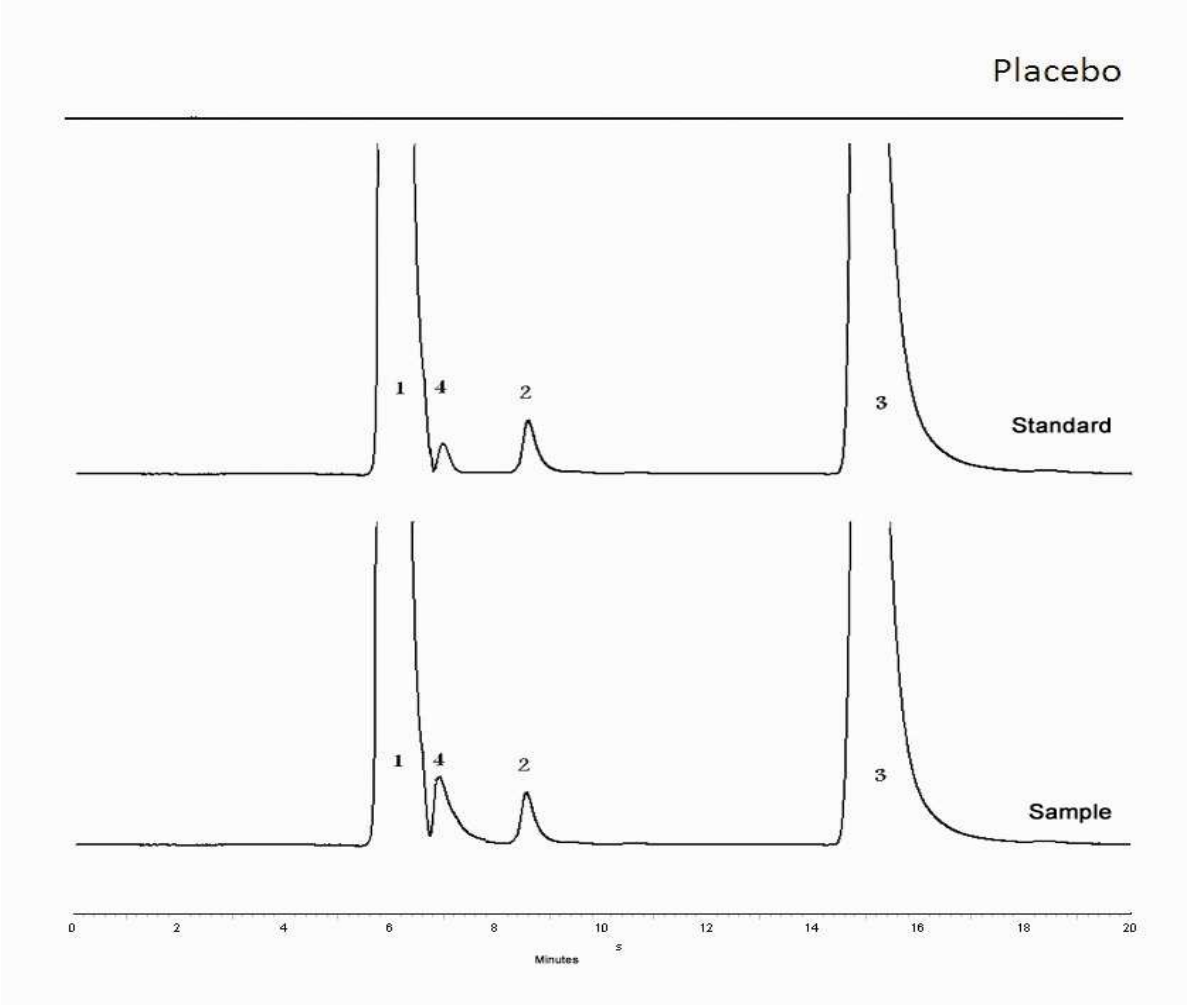


Figure (5.2): A zoomed out view chromatograms of Placebo, standard solution of sulfaquinoxaline sodium, vitamin K_3 and amprolium hydrochloride and test solution of A.S.K powder ; Column, a ZIC-HILIC column (25.0 cm long ×4.6 mm i.d.) with particle size of 5 μm protected with a ZIC-HILIC guard column (20mm× 2.1mm, 5μm); Mobile phase, acetonitrile and 0.2M ammonium acetate solution (15:85; v/v) adjusted to pH 5.7; flow rate 0.5 ml/min; λ =263nm; column temperature=25°C; Autosampler Temp. = 15°C; concentration of vitamin K_3 was about 0.2μg/ml.

5.2 Linearity

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).

5.2.1. Linearity for sulfaquinoxaline sodium:

The linearity for sulfaquinoxaline sodium in the range of 12µg/ml to 26µg/ml (60% to 130% of the labeled amount) was investigated. Data and statistical evaluation are listed in table 5.1 and figure 5.3

Table (5.1): Linearity results and statistical evaluation for sulfaquinoxaline sodium in the range of 12µg/ml to 26µg/ml (60% to 130%)

Conc. (µg/ml)	**Sample peak area**			
	Inj.1	**Inj.2**	**Inj.3**	**Average**
12.0	7158529	7189186	7146490	7164735
16.0	9567483	9574101	9652688	9613395
20.0	11812875	11778166	11822609	11800388
24.0	14318735	14280983	14374688	14327836
26.0	15568277	15577638	15567050	15572344
Average Area				11695739
Statistical data for regression line				
Parameter		**Results**		**A.C.***
n*		15		----
Coefficient of correlation		0.99973		Min. 0.995
R^2		0.9995		NLT 0.99
Y-Intercept		-11291= -0.1%		$\leq \pm$ 2% of the average area

Where,

***n:** number of determinations

***A.C.:** Acceptance criteria

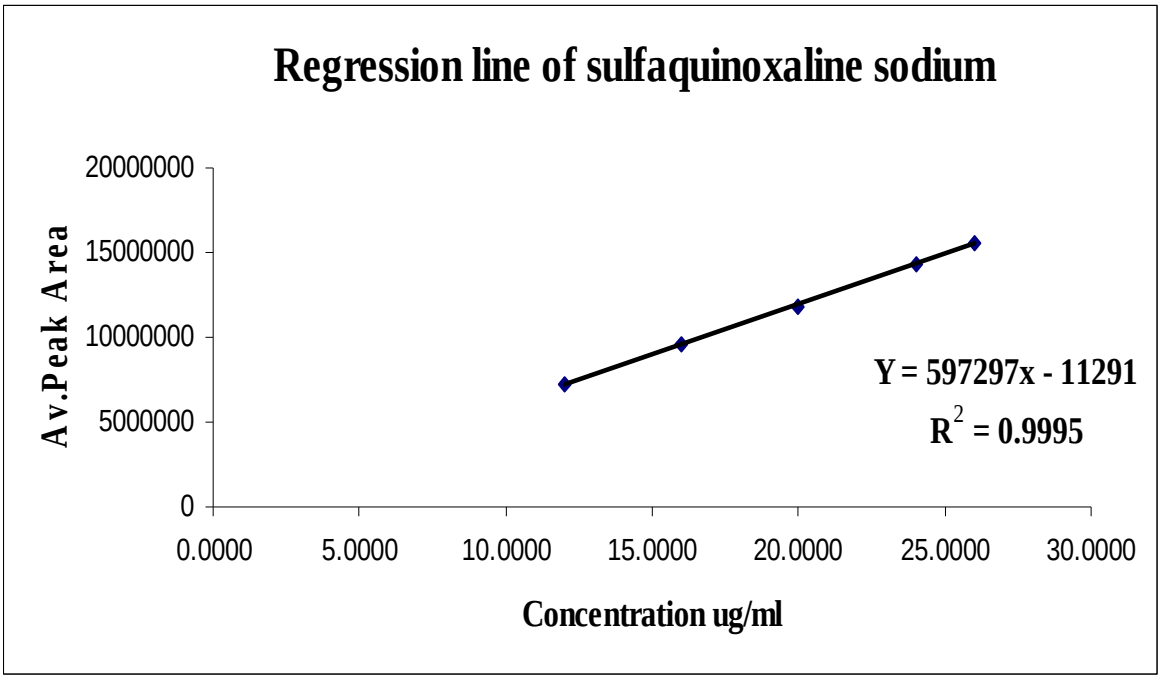


Figure (5.3): The regression analysis of sulfaquinoxaline sodium over the range of 60% to 130% of labeled amount (12µg/ml to 26µg/ml)

Assessment:

The regression line of sulfaquinoxaline sodium demonstrates linearity in the range of 60% to 130% (figure 5.3). The regression analysis (table 5.1) confirms that the deviation of the y-intercept from zero is not significant, the regression line is linear, $\mathbf{R^2}$ = 0.9995> 0.99 ; thus the method is linear in the range of 60% to 130% of the labeled amount, as shown in figure 5.3 and table 5.1.

5.2.2. Linearity of Amprolium hydrochloride:

The linearity for amprolium hydrochloride in the range of 12µg/ml to 26µg/ml (60% to 130% of the labeled amount) was investigated. Data and statistical evaluation are listed in table 5.2 and figure 5.4

Table (5.2): Linearity results and statistical evaluation for amprolium hydrochloride in the range of 12µg/ml to 26µg/ml (60% to 130%)

Conc. (µg/ml)	Sample peak area			
	Inj.1	**Inj.2**	**Inj.3**	**Average**
12	3277273	3292404	3267086	3278921
16	4359249	4363904	4364580	4364242
20	5512413	5501599	5501665	5501632
24	6502384	6505952	6516029	6510991
26	7042134	7055996	7043506	7049751
Average Area				5341107

Statistical data for regression line		
Parameter	**Results**	**A.C.**
n	15	----
Coefficient of correlation	0.99981	Min. 0.995
R^2	0.9996	NLT 0.99
Y-Intercept	62202 = 1.16 %	$\leq \pm$ 2% of the average area

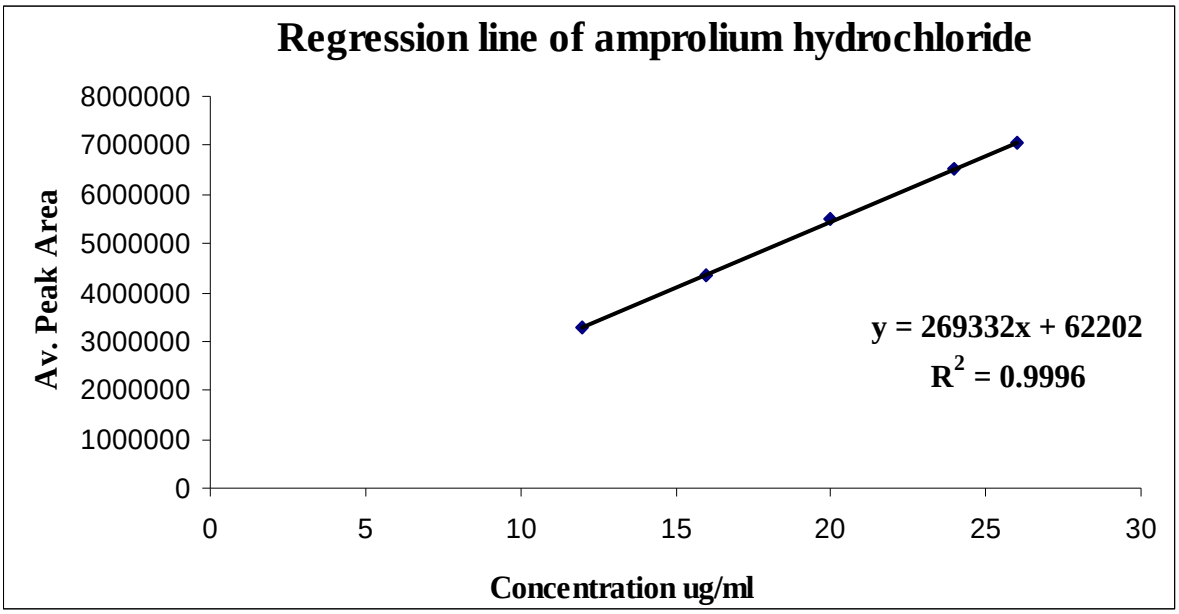


Figure (5.4): The regression analysis of amprolium hydrochloride over the range of 60% to 130% of labeled amount (12µg/ml to 26µg/ml)

Assessment:

The regression line of amprolium hydrochloride demonstrates linearity in the range of 60% to 130% (figure 5.4). The regression analysis (table 5.2) confirms that the deviation of the y-intercept from zero is not significant, the regression line is linear, $\mathbf{R^2}$ = 0.9996> 0.99; thus the method is linear in the range of 60% to 130% of the labeled amount, as shown in figure 5.4 and table 5.2.

5.2.3. Linearity of Menadione (Vitamin K_3):

The linearity for menadione (as sodium bisulfite) in the range of 0.12µg/ml to 0.26µg/ml (60% to 130% of the labeled amount) was investigated. Data and statistical evaluation are listed in table 5.3 and figure 5.5

Table (5.3): Linearity results and statistical evaluation for menadione (as sodium bisulfite) in the range of 0.12µg/ml to 0.26µg/ml (60% to 130%) of labeled amount.

Conc. (µg/ml)	Sample peak area			
	Inj.1	**Inj.2**	**Inj.3**	**Average**
0.120	67453	67704	67507	67555
0.160	87768	87861	87810	87836
0.200	108379	106955	108130	107543
0.240	132793	132358	133828	133093
0.260	145865	145003	145638	145321
Average Area				108269

Statistical data for regression line		
Parameter	**Results**	**A.C.**
n	15	----
Coefficient of correlation	0.99833	NLT 0.995
R^2	0.9967	NLT 0.99
Y-Intercept	-644.34 = -0.6%	$\leq \pm$ 2% of the average area

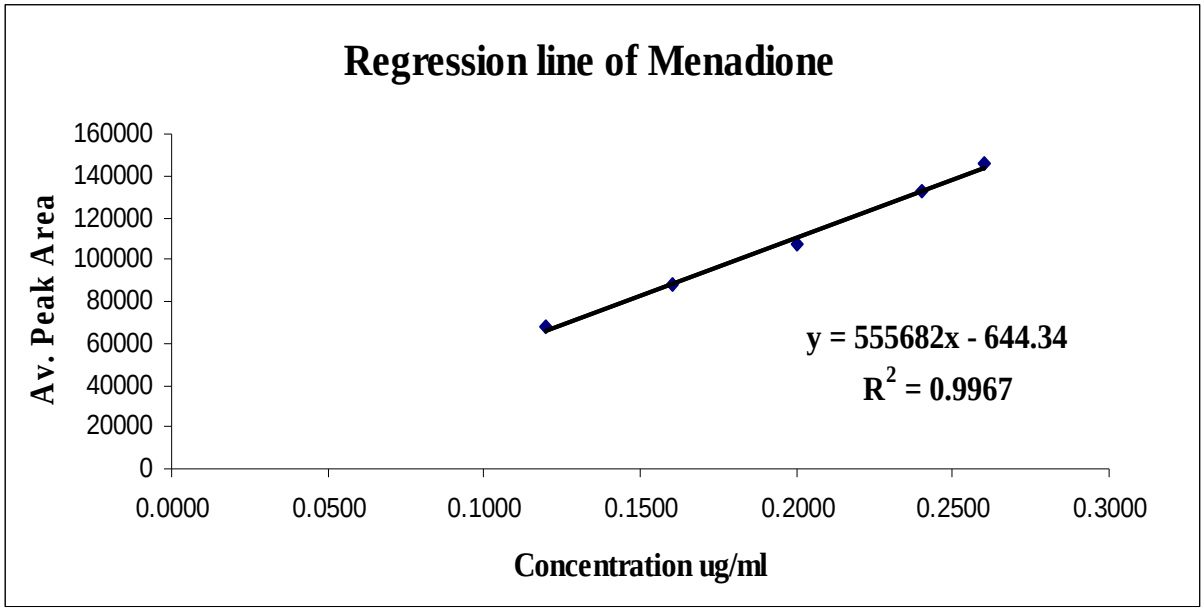


Figure (5.5): The regression analysis of menadione (as sodium bisulfite) over the range of 60% to 130% of labeled amount (0.12µg/ml to 0.26µg/ml).

Assessment:

The regression line of menadione (as sodium bisulfite) demonstrates linearity in the range of 60% to 130% (figure 5.5). The regression analysis (table 5.3) confirms that the deviation of the y-intercept from zero is not significant, the regression line is linear, $\mathbf{R^2}$ = 0.9967> 0.99; thus the method is linear in the range of 60% to 130% of the labeled amount, as shown in figure 5.5 and table 5.3.

5.3 Range

Considering the data for the regression line of sulfaquinoxaline sodium, amprolium hydrochloride, and menadione (as sodium bisulfite), linearity of each is given in the range of 60% to 130% of the labeled amount of final concentration.

5.4 Accuracy (recovery)

The data obtained for the evaluation of the linearity were used. In all the experiments for accuracy test, the different concentrations of the components mixture were added to placebo matrix and the accuracy was measured as reflected by recovery as shown in the below formula:

$$\% \text{ Recovery} = (\text{Amount found}/\text{Amount added}) \times 100$$

5.4.1. Accuracy (recovery) for sulfaquinoxaline sodium:

The data obtained for the evaluation of the linearity were used. The accuracy as reflected from recovery data and statistical evaluation for assay of sulfaquinoxaline sodium over the range of 12µg/ml to 26µg/ml (60% to 130% of the specified final concentration) is listed in table 5.4.

Table (5.4): Recovery data and statistical evaluation of sulfaquinoxaline sodium over the range of 60% to 130% of the labeled amount of final concentration

Concentration level %	1st Value (%)	2nd Value (%)	3rd Value (%)
60	99.2	99.5	99.0
80	99.4	99.5	100.3
100	98.2	97.9	98.3
120	99.2	98.9	99.6
130	99.5	99.6	99.5

Statistical data		
Parameter	**Results**	**A.C. ***
n	15	-----
$\overline{X}$	99.2	96.0-104.0%
S.D.	0.63	-----
C.V. (%)	0.63	$\leq$ 2.0
***C.I. (P=95%)**	98.8% to 99.6%	96.0-104.0%

***C.I.:** Confidence interval of the average value (P=95%) and is calculated by the following formula:

$$C.I. = \overline{X} \pm (t \times S.D/\sqrt{n})$$

$\overline{X}$ **:** The average of recovery values

t: A parameter that depends upon the number of degrees of freedom and the confidence level required.

S.D.: Standard deviation

n: Number of determinations

Assessment:

The recovery shows results between 97.9% and 100.3 with coefficient of variation of 0.63 %. The acceptance criteria are fulfilled.

5.4.2. Accuracy (recovery) for amprolium hydrochloride:

The data obtained for the evaluation of the linearity were used. The recovery data and statistical evaluation for assay of amprolium hydrochloride over the range of 12µg/ml to 26µg/ml (60% to 130% of the specified final concentration) are listed in table 5.5.

Table (5.5): Recovery data and statistical evaluation of amprolium hydrochloride over the range of 60% to 130% of the labeled amount of final concentration

Concentration level %	**1st Value (%)**	**2nd Value (%)**	**3rd Value (%)**
60	98.9	99.3	98.6
80	98.6	98.7	98.8
100	99.8	99.6	99.6
120	98.1	98.1	98.3
130	98.1	98.2	98.1
Statistical data			
Parameter	**Results**	**A.C.**	
n	15	-----	
$\overline{X}$	99.0	96.0-104.0%	
S.D.	0.59	-----	
C.V. (%)	0.6	$\leq$ 2.0	
C.I. (P=95%)	98.7% to 99.3%	96.0-104.0%	

Assessment: The recovery shows results between 98.1% and 99.8% with coefficient of variation of 0.6%. The acceptance criteria are fulfilled.

5.4.3. Accuracy (recovery) for menadione (as sodium bisulfite):

The data obtained for the evaluation of the linearity were used. The recovery data and statistical evaluation for assay of amprolium hydrochloride over the range of 0.12µg/ml to 0.26µg/ml (60% to 130% of the specified final concentration) are listed in table 5.6.

Table (5.6): Recovery data and statistical evaluation of menadione (as sodium bisulfite) over the range of 60% to 130% of the labeled amount of final concentration

Concentration level %	1st Value (%)	2nd Value (%)	3rd Value (%)
60	101.9	102.3	102.0
80	99.5	99.6	99.5
100	98.3	97.0	98.0
120	100.3	100.0	101.1
130	101.7	101.1	101.6
Statistical data			
Parameter	**Results**	**A.C.**	
n	15	-----	
$\overline{X}$	100.3	96.0-104.0%	
S.D.	1.61	-----	
C.V. (%)	1.61	$\leq$ 3.0	
C.I. (P=95%)	99.4% to 101.2%	96.0-104.0%	

Assessment:

The recovery shows results between 97.0 % and 102.3% with coefficient of variation of 1.61 % .The acceptance criteria are fulfilled.

5.5 Precision

5.5.1. Precision of sulfaquinoxaline sodium:

5.5.1.1. Repeatability:

One laboratory analyst carried out the assay of sulfaquinoxaline sodium on six determinations of homogeneous sample of A.S.K powder Batch Number: F01211ER at 100% of the test concentration with the same analytical equipment at the same day. The results are listed on table 5.7.

Table (5.7): Assay results and Statistical evaluation for repeatability of sulfaquinoxaline sodium in A.S.K powder Batch Number: F01211ER

n=6	**Assay of sulfaquinoxaline sodium (%)**	
1	98.36	
2	99.22	
3	99.07	
4	99.14	
5	97.52	
6	99.66	
Statistical data		
Parameter	**Results**	**A.C.**
$\overline{X}$	98.83	90.0 -110%
S.D.	0.76	----
C.V. (%)	0.77	≤ 2 %
C.I. (P=95%)	98.0% to 99.7%	96.0-104.0%

Assessment: The coefficient of variation of 0.77 % shows that the results of the HPLC assay procedure for determining sulfaquinoxaline sodium is within a suitable repeatability for the specified range. The acceptance criteria are fulfilled.

5.5.1.2. Intermediate Precision (ruggedness):

Two laboratory analysts carried out the assay of sulfaquinoxaline sodium on 12 homogeneous samples of A.S.K powder at 100% of the final test concentration, with different analytical equipments at different days. The results are listed on table 5.8.

Table (5.8): Assay results and Statistical evaluation for intermediate precision of sulfaquinoxaline sodium in A.S.K powder Batch Number: F01211ER

n=12	1st assay of Sulfaquinoxaline sodium	2nd assay of Sulfaquinoxaline sodium
1	98.36	97.67
2	99.22	97.89
3	99.07	98.77
4	99.14	97.69
5	97.52	99.13
6	99.66	98.80
Statistical data		
Parameter	**Results**	**A.C.**
$\overline{X}$	98.58	90.0 -110%
S.D.	0.71	----
C.V. (%)	0.72	≤ 3 %
C.I. (P=95%)	98.1% to 99.0%	96.0-104.0%

Assessment:

The coefficient of variation of 0.72% shows that the results of the HPLC assay procedure for determining sulfaquinoxaline sodium is within a suitable intermediate precision for the specified range. The acceptance criteria are fulfilled.

5.5.2. Precision of amprolium hydrochloride:

5.5.2.1. Repeatability:

One laboratory analyst carried out the assay of amprolium hydrochloride on six determinations of homogeneous sample of A.S.K powder at 100% of the test concentration with the same analytical equipment at the same day. The results are listed on table 5.9.

Table (5.9): Assay results and Statistical evaluation for repeatability of amprolium hydrochloride in A.S.K powder Batch Number: F01211ER

n=6	Assay of amprolium hydrochloride (%)	
1	99.37	
2	99.38	
3	99.89	
4	100.24	
5	99.50	
6	101.04	
Statistical data		
Parameter	**Results**	**A.C.**
$\overline{X}$	99.90	90.0 -110%
S.D.	0.65	----
C.V. (%)	0.65	$\leq$ 2 %
C.I. (P=95%)	99.2% to 100.6%	96.0-104.0%

Assessment:

The coefficient of variation of 0.59 % shows that the results of the HPLC assay procedure for determining amprolium hydrochloride is within a suitable repeatability for the specified range. The acceptance criteria are fulfilled.

5.5.2.2. Intermediate Precision (ruggedness):

Two laboratory analysts carried out the assay of amprolium hydrochloride on 12 homogeneous samples of A.S.K powder at 100% of the final test concentration, with different analytical equipments at different days. The results are listed on table 5.10.

Table (5.10): Assay results and Statistical evaluation for intermediate precision of amprolium hydrochloride assay in A.S.K powder Batch Number: F01211ER

n=12	1st assay of Amprolium hydrochloride	2nd assay of Amprolium hydrochloride
1	99.37	101.76
2	99.38	101.26
3	99.89	101.67
4	100.24	100.53
5	99.50	102.24
6	101.04	101.07
Statistical data		
Parameter	**Result**	**A.C.**
$\overline{X}$	100.66	90.0 -110%
S.D.	0.624	----
C.V. (%)	0.62	≤ 3 %
C.I. (P=95%)	100.3% to 101.1%	96.0-104.0%

Assessment:

The coefficient of variation 0.62 % shows that the results of the HPLC assay procedure for determining amprolium hydrochloride is within a suitable intermediate precision for the specified range. The acceptance criteria are fulfilled.

5.5.3. Precision of menadione (as sodium bisulfite):

5.5.3.1. Repeatability:

One laboratory analyst carried out the assay of menadione on six determinations of homogeneous sample of A.S.K powder at 100% of the test concentration with the same analytical equipment at the same day. The results are listed on table 5.11.

Table (5.11): Assay results and Statistical evaluation for repeatability of menadione in A.S.K powder Batch Number: F01211ER

n=6	**Assay of menadione (%)**	
1	97.70	
2	101.48	
3	101.11	
4	102.70	
5	97.94	
6	98.59	
Statistical data		
Parameter	**Result**	**A.C.**
$\overline{X}$	99.92	90.0 -110%
S.D.	2.1	----
C.V. (%)	2.1	≤ 3 %
C.I. (P=95%)	97.5% to 102.4%	96.0-104.0%

Assessment:

The coefficient of variation of 2.1 % shows that the results of the HPLC assay procedure for determining menadione is within a suitable repeatability for the specified range. The acceptance criteria are fulfilled.

5.5.3.2. Intermediate Precision (ruggedness):

Two laboratory analysts carried out the assay of menadione on 12 homogeneous samples of A.S.K powder at 100% of the final test concentration, with different analytical equipments at different days. The results are listed on table 5.12.

Table (5.12): Assay results and Statistical evaluation for intermediate precision of menadione in A.S.K powder Batch Number: F01211ER

Number of determination n=12	**1st assay of Menadione**	**2nd assay of Menadione**
1	97.70	98.87
2	101.48	100.03
3	101.11	100.52
4	102.70	99.01
5	97.94	100.81
6	98.59	100.91
Statistical data		
Parameter	**Result**	**A.C.**
$\overline{X}$	99.97	90.0 -110%
S.D.	1.5	----
C.V. (%)	1.5	≤ 3 %
C.I. (P=95%)	99.0% to 100.9%	96.0-104.0%

Assessment:

The coefficient of variation 1.5 % shows that the results of the HPLC assay procedure for determining menadione is within a suitable intermediate precision for the specified range. The acceptance criteria are fulfilled.

5.6 Robustness

5.6.1. Stability of standard & sample solution:

5.6.1.1. Stability of the standard solution (stored at 15°C):

The stability of the standard solution at temperature of 15°C stored in closed volumetric flask protected from light during a period of 24 hours is investigated. Data and statistical evaluation are listed in table 5.13.

Table (5.13): Results and statistical evaluation of the assay of sulfaquinoxaline sodium, amprolium hydrochloride and menadione standard solution during a period of 24 hours.

Time (Hours)	**sulfaquinoxaline sodium (Av.PAV)**	**amprolium hydrochloride (Av.PAV)**	**Menadione (Av.PAV)**
0	11961069.33	5526863.333	111602.3333
24	12008352.00	5485563.667	112757.3333
Statistical data			
Parameter	**sulfaquinoxaline sodium**	**amprolium hydrochloride**	**Menadione**
n	2	2	2
$\overline{X}$	11984710.67	5506213.5	112179.8333
ΔAv.PAV *100	0.4 %	0.8%	1.0%
A.C.	≤2.0%	≤2.0%	≤3.0%

***ΔAv.PAV :** The difference between average peak area value after 24 hours and at 0 time divided by average peak area at 0 time.

$$\Delta Av.PAV = [Av.PAV\ (t=24hr) - Av.PAV(t=0)] / Av.PAV(t=0)$$

Assessment:

The Av. PAV of sulfaquinoxaline sodium, amprolium hydrochloride and menadione remained stable. Therefore the standard solution can be stored during a period of 24 hours at the temperature 15°C for the assay test.

5.6.1.2. Stability of the sample solution (stored 15°C):

The stability of the sample solution at temperature of 15°C stored in closed volumetric flask protected from light during a period of 24 hours is investigated. Data and statistical evaluation are shown in table 5.14.

Table (5.14): Results and statistical evaluation for the assay of sulfaquinoxaline sodium, amprolium hydrochloride and menadione sample solution during a period of 24 hours.

Time (Hours)	sulfaquinoxaline sodium (Av.PAV)	amprolium hydrochloride (Av.PAV)	Menadione (Av.PAV)
0	11870856	5626206.5	105886
24	11785329.5	5593480.5	106162
Statistical data			
Parameter	**sulfaquinoxaline sodium**	**amprolium hydrochloride**	**Menadione**
n	2	2	2
$\overline{X}$	11828092.75	5609843.5	106024
∆Av.PAV *100	0.7 %	0.6%	0.3%
A.C.	≤3.0%	≤3.0%	≤3.0%

Assessment:

The Av. PAV of sulfaquinoxaline sodium, amprolium hydrochloride and menadione remained stable. Therefore the sample solution can be stored during a period of 24 hours at the temperature 15°C for the assay test.

5.6.2. Different ammonium acetate buffer percent in mobile phase:

The sample and standard using different buffer percentages in the mobile phase (-3.0, +3.0) and normal % value was investigated. The results are listed in Table 5.15.

Table (5.15): Assay results and statistical evaluation for sulfaquinoxaline sodium, amprolium hydrochloride and menadione using different % of buffer in mobile phase (-3.0, +3.0) and normal % value.

Mobile phase variation(v/v) ACN/0.2M ammonium acetate	**% Assay of sulfaquinoxaline sodium**	**% Assay of amprolium hydrochloride**	**% Assay of menadione**
88:12	99.3	99.9	101.5
85:15	101.6	101.3	102.6
82:18	101.4	101.9	101.3
Statistical data			
Parameter	**sulfaquinoxaline sodium**	**amprolium hydrochloride**	**Menadione**
n	3	3	3
$\overline{X}$	100.8	101.0	101.8
S.D.	1.27	1.03	0.7
C.V. (%)	1.26	1.02	0.7
A.C.	≤2.0%	≤2.0%	≤3.0%

Assessment: Variation of ammonium acetate buffer in the range of (± 3 of the nominal value) shows no significant change in the final assay results of each of the above three compounds.

5.6.3. Different column batches (lots):

Two column batches filled with the prescribed stationary phases were investigated. The results are listed in Table 5.16.

Table (5.16): Assay results and statistical evaluation for sulfaquinoxaline sodium, amprolium hydrochloride and menadione using two different column batches

Column batch Number	**% Assay of sulfaquinoxaline sodium**	**% Assay of amprolium hydrochloride**	**% Assay of menadione**
L010134877	101.6	101.5	101.3
L010129977	101.6	101.3	102.6
Statistical data			
Parameter	**sulfaquinoxaline sodium**	**amprolium hydrochloride**	**Menadione**
n	2	2	2
$\overline{X}$	101.6	101.4	101.95
S.D.	0	0.4	0.9
C.V. (%)	0	0.4	0.9
A.C.	≤2.0%	≤2.0%	≤3.0%

Assessment:

Using different column batches shows no significant change in the final assay results of each of the above three compounds.

5.6.4. Variations of the column temperature:

Three different column temperatures were investigated in the range 23-27°C. The results are listed in table 5.17.

Table (5.17): Assay results and statistical evaluation for sulfaquinoxaline sodium, amprolium hydrochloride and menadione using different column temperatures

Column Temp. (°C)	**% Assay of sulfaquinoxaline sodium**	**% Assay of amprolium hydrochloride**	**% Assay of menadione**
23	99.6	99.9	100.0
25	101.6	101.5	101.3
27	100.2	100.9	100.6
Statistical data			
Parameter	**sulfaquinoxaline sodium**	**amprolium hydrochloride**	**Menadione**
n	3	3	3
$\overline{X}$	100.3	100.8	100.6
S.D.	1.02	0.8	0.65
C.V. (%)	1.02	0.8	0.64
A.C.	≤2.0%	≤2.0%	≤3.0%

Assessment:

Using different column temperatures in the range of 23-27°C shows no significant change in the final assay results of each of the above three compounds.

5.6.5. Use of Different flow rates:

Different mobile phase flow rates of 0.45, 0.50, and 0.55 ml/min. were investigated. The results are shown in Table 5.18.

Table (5.18): Assay results and statistical evaluation for sulfaquinoxaline sodium, amprolium hydrochloride and menadione using three different flow rates.

F.R. (ml/min)	% Assay of sulfaquinoxaline sodium	% Assay of amprolium hydrochloride	% Assay of menadione
0.45	100.57	100.97	99.5
0.50	101.6	101.5	101.3
0.55	100.45	100.51	100.50
Statistical data			
Parameter	**sulfaquinoxaline sodium**	**amprolium hydrochloride**	**Menadione**
n	3	3	3
$\overline{X}$	100.87	101.0	100.43
S.D.	0.6	0.5	0.9
C.V. (%)	0.6	0.5	0.9
A.C.	≤2.0%	≤2.0%	≤3.0%

Assessment:

Using different flow rates in the range of 0.45 to 0.55 ml/min. shows no significant change in the final assay results of each of the above three compounds.

5.6.6. Variation of mobile phase pH values:

Three different pH values of the mobile phase (5.6, 5.7, and 5.8) were investigated. The results are listed in Table 5.19.

Table (5.19): Assay results and statistical evaluation for sulfaquinoxaline sodium, amprolium hydrochloride and menadione using three different mobile phase pH values

pH	% Assay of sulfaquinoxaline sodium	% Assay of amprolium hydrochloride	% Assay of menadione
5.6	101.96	100.44	101.12
5.7	100.6	101.34	101.42
5.8	101.33	101.14	100.16
Statistical data			
Parameter	**sulfaquinoxaline sodium**	**amprolium hydrochloride**	**Menadione**
n	3	3	3
$\overline{X}$	101.3	100.97	100.9
S.D.	0.7	0.5	0.7
C.V. (%)	0.7	0.5	0.7
A.C.	≤2.0%	≤2.0%	≤3.0%

Assessment:

Using mobile phase pH in the range of 5.7 ± 0.1 shows no significant change in the final assay results of each of the above three compounds.

5.7 Stress Tests

The excipients and active substances were submitted to acidic, basic, oxidizing, light and dry heat forced degradation conditions. Samples were analyzed by HPLC to proof the stability indicating capability. The degradation peaks detected in the chromatogram using the HPLC method are completely separated and no interference was detected whatever the stressed conditions are.

One may also conclude that the method developed for determination of sulfaquinoxaline sodium, amprolium hydrochloride and menadione (as sodium bisulfite) is stability indicating and thus can be used for the determination of sulfaquinoxaline sodium, amprolium hydrochloride and menadione (as sodium bisulfite) in A.S.K powder for the control of the finished product and for the stability testing.

5.7.1. Stress tests for excipient:

Separate samples of the excipient (dextrose monohydrate) were treated as follows:

The 1st sample: About 59.8 mg of the dextrose monohydrate is heated for 72 hours at 105°C in an oven and then dissolved in 100 ml 90% ACN. Finally, 5 ml of this solution is diluted to 50 ml with mobile phase.

The 2nd sample: About 59.8 mg of the dextrose monohydrate is mixed with 20 ml of 1 M HCl for 24 hours, neutralized with 1 M NaOH and then diluted to 100 ml with 90% ACN. Finally, 5 ml of this solution is diluted to 50 ml with mobile phase.

The 3rd sample: About 59.8 mg of the dextrose monohydrate is mixed with 20 ml of 1 M NaOH for 24 hours, neutralized with 1 M HCl and then diluted to 100 ml with 90% ACN. Finally, 5 ml of this solution is diluted to 50 ml with mobile phase.

The 4th Sample: About 59.8 mg of the dextrose monohydrate is mixed with 95 ml of 3% H_2O_2 for 1 hour and then diluted to 100 ml with 90% ACN. Finally, 5 ml of this solution is diluted to 50 ml with mobile phase.

The 5th sample: About 59.8 mg of the dextrose monohydrate is stressed under day light for 72 hours and then dissolved in 100 ml 90% ACN. Finally, 5 ml of this solution is diluted to 50 ml with mobile phase.

Figure 5.6 illustrates clearly that the excipient did not generate any degradation products under any of the above stress conditions except under H_2O_2 which give a peak with a retention time of about 10 minutes that is due to H_2O_2 and it is not due to degradation.

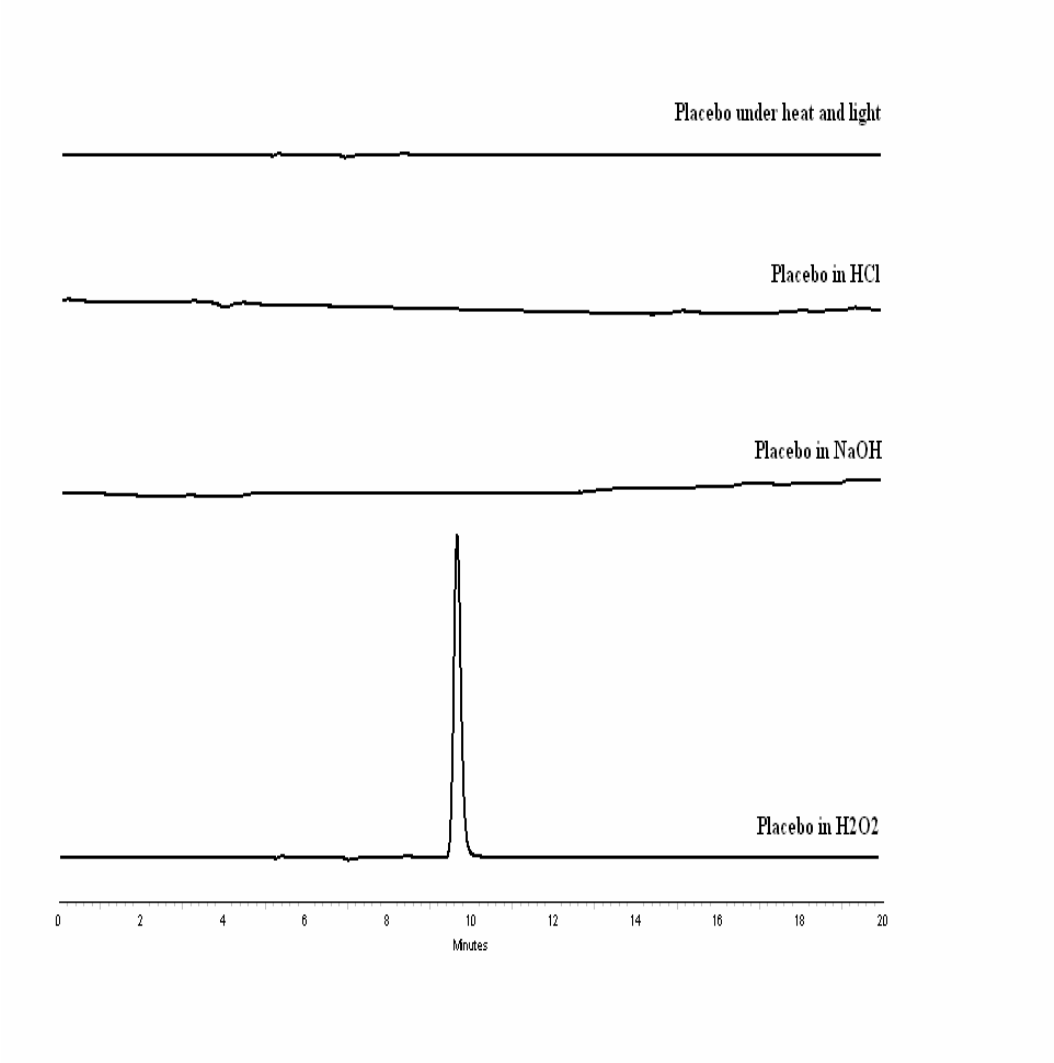


Figure (5.6): Stress tests for excipient

5.7.2. Stress tests for sulfaquinoxaline sodium:

5.7.2.1. Stress test with heat (105°C):

20 mg of Sulfaquinoxaline sodium was heated for 72 hours at 105°C in an oven and then dissolved in 100 ml 90% ACN. Finally, 5 ml of this solution is diluted to 50 ml with mobile phase.

Figure 5.7 illustrates clearly that sulfaquinoxaline sodium under heat stress conditions shows a slight increase of known potential degradation impurity with relative retention time (RRT) of about 1.2 related to sulfaquinoxaline sodium. It is worthwhile mentioning that this impurity is the very same impurity appeared in our A.S.K powder.

Assessment:

The potential degradation product (impurity) of the sulfaquinoxaline sodium was observed under stress test with heat. The degradation impurity is well separated from the sulfaquinoxaline sodium and other active ingredients. Peak (1) is sulfaquinoxaline and peak (2) is due to its impurity (sulfaquinoxaline impurity A).

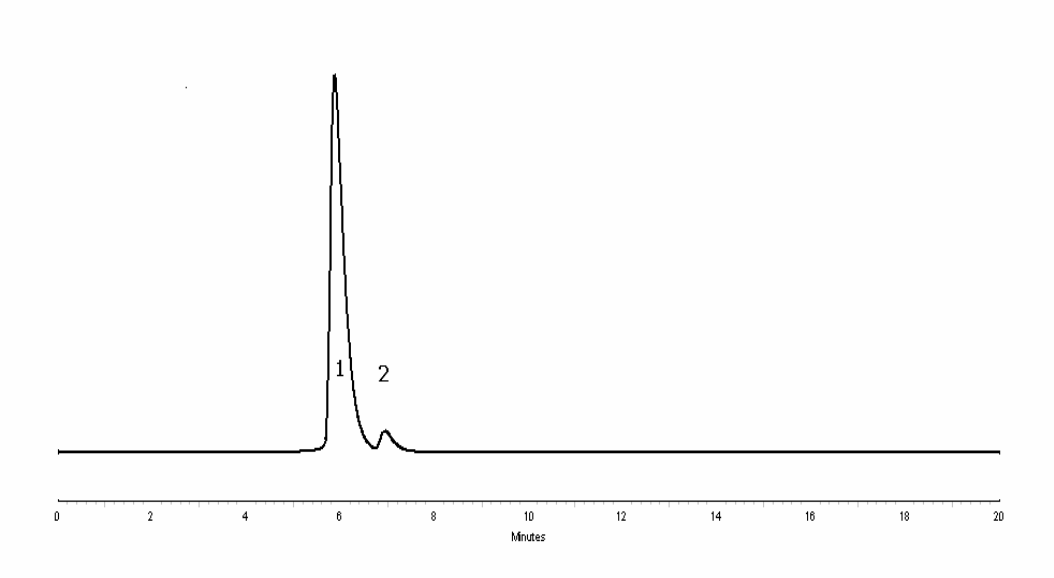


Figure (5.7): Stress test with heat for sulfaquinoxaline sodium

5.7.2.2. Stress test under oxidation:

20 mg of sulfaquinoxaline sodium was mixed with 95 ml of 3% H_2O_2 for one hour and then diluted to 100 ml with 90% ACN. Finally, 5 ml of this solution is diluted to 50 ml with mobile phase.

Figure 5.8 illustrates clearly that sulfaquinoxaline sodium under stress conditions with 3% H_2O_2 shows a slight increase of two potential degradation impurities with relative retention times (RRT) of 1.2 and 1.3 related to sulfaquinoxaline sodium. It is worthwhile mentioning that impurity with RRT of 1.2 is the very same impurity appeared in our A.S.K powder.

Assessment:

The potential degradation products (impurities) of the sulfaquinoxaline sodium were observed under stress test with oxidation. The degradation impurities are well separated from the sulfaquinoxaline sodium and other active ingredients. Peak (1) is sulfaquinoxaline and other peaks (2) and (3) are due to degradation impurities. Peak (4) with relative retention time of 1.7 is due to H_2O_2 (was observed in the placebo).

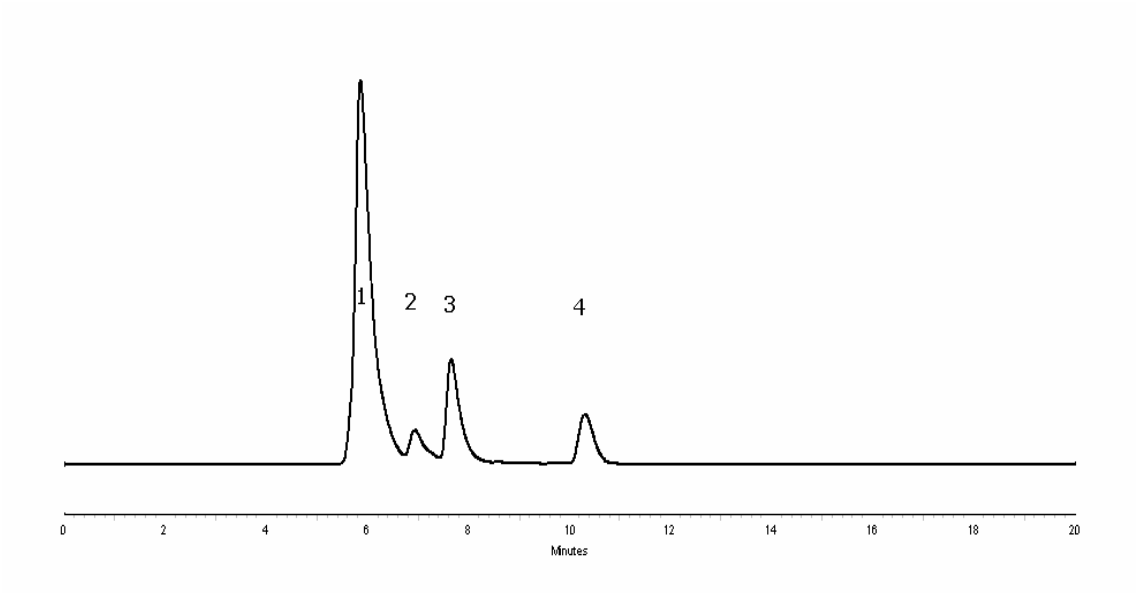


Figure (5.8): Stress test under oxidation for sulfaquinoxaline sodium

5.7.2.3. Stress test with light:

20 mg of sulfaquinoxaline sodium was stressed under day light for 72 hours and then dissolved in 100 ml 90% ACN. Finally, 5 ml of this solution is diluted to 50 ml with mobile phase.

Figure 5.9 illustrates clearly that sulfaquinoxaline sodium under light stress conditions shows a slight increase of known potential degradation impurity (sulfaquinoxaline impurity A) with relative retention time of about 1.2 related to sulfaquinoxaline sodium.

Assessment:

The potential degradation product (impurity) of the sulfaquinoxaline sodium was observed under stress test with light. The degradation impurity is well separated from the sulfaquinoxaline sodium and other active ingredients. Peak (1) is sulfaquinoxaline and peak (2) is due to its impurity (sulfaquinoxaline impurity A).

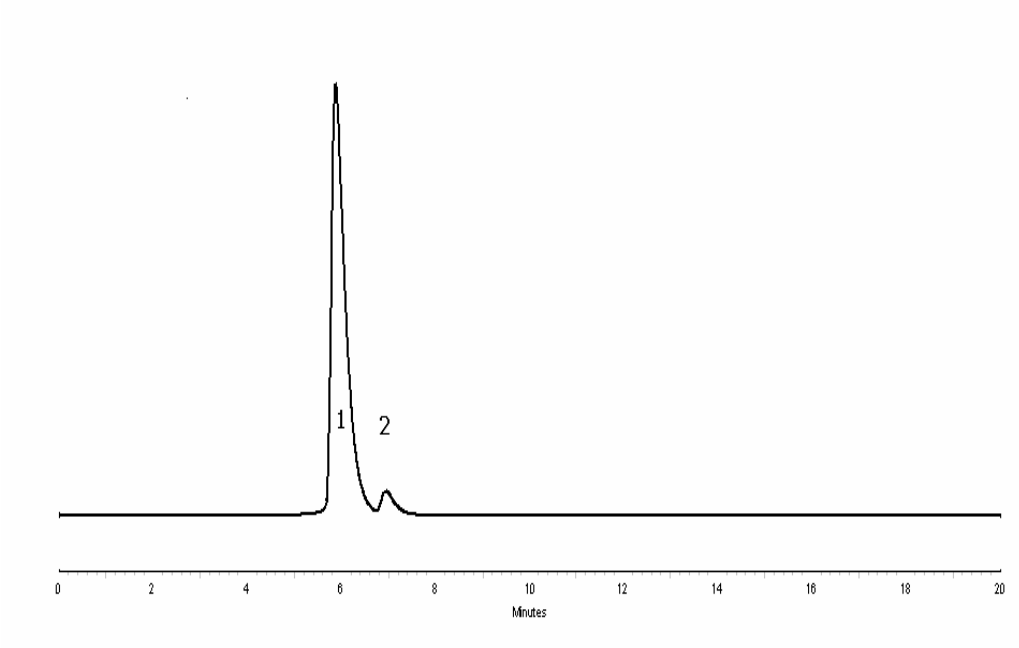


Figure (5.9): Stress test with light for sulfaquinoxaline sodium

5.7.2.4. Stress test with hydrochloric acid:

20 mg of sulfaquinoxaline sodium was mixed with 20 ml of 1 M HCl for 24 hours, neutralized with 1M NaOH and then diluted to 100 ml with 90% ACN. Finally, 5 ml of this solution is diluted to 50 ml with mobile phase.

Figure 5.10 illustrates clearly that sulfaquinoxaline sodium under stress conditions with HCl shows a slight increase of a known potential degradation impurity (sulfaquinoxaline impurity A) with a relative retention time of about 1.2 related to sulfaquinoxaline sodium.

Assessment:

The potential degradation product (impurity) of the sulfaquinoxaline sodium was observed under stress test with HCl. The degradation impurity is well separated from the sulfaquinoxaline sodium and other active ingredients. Peak (1) is sulfaquinoxaline and peak (2) is due to its impurity.

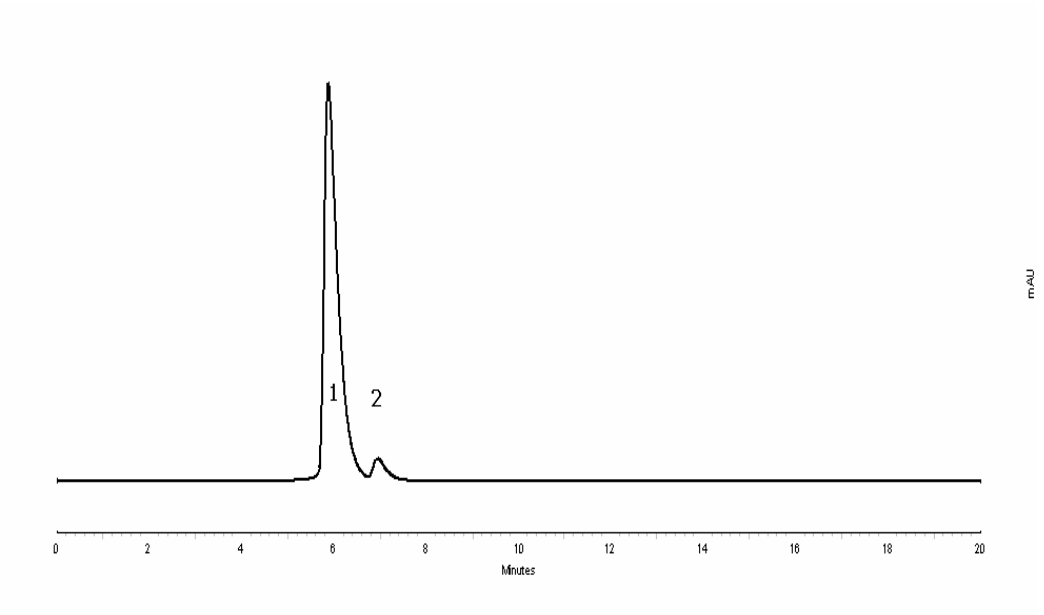


Figure (5.10): Stress test with hydrochloric acid for sulfaquinoxaline sodium.

5.7.2.5. Stress test with Sodium Hydroxide:

20 mg of sulfaquinoxaline sodium was mixed with 20 ml of 1 M NaOH for 24 hours, neutralized with 1M HCl and then diluted to 100 ml with 90% ACN. Finally, 5 ml of this solution is diluted to 50 ml with mobile phase.

Figure 5.11 illustrates clearly that sulfaquinoxaline sodium under stress conditions with sodium hydroxide shows a slight increase of a known potential degradation impurity (sulfaquinoxaline impurity A) with relative retention time of about 1.2 related to sulfaquinoxaline sodium.

Assessment:

The potential degradation product (impurity) of the sulfaquinoxaline sodium was observed under stress test with NaOH. The degradation impurity is well separated from the sulfaquinoxaline sodium and other active ingredients. Peak (1) is sulfaquinoxaline and peak (2) is due to its impurity.

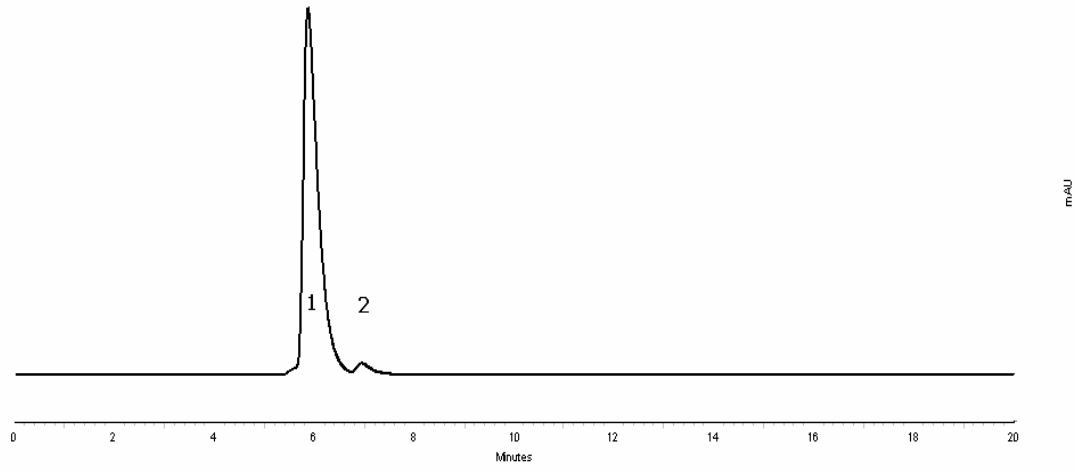


Figure (5.11): Stress test with sodium hydroxide for sulfaquinoxaline sodium

5.7.2. Stress tests for Menadione (as sodium bisulfite):

5.7.2.1. Stress test with heat (105°C):

32 mg menadione sodium bisulfite was heated for 72 hours at 105°C in an oven and then dissolved in 100 ml 90% ACN. Finally, 5 ml of this solution is diluted to 50 ml with mobile phase.

Figure 5.12 illustrates clearly that menadione sodium bisulfite under heat stress conditions shows an increase on unknown potential degradation impurity with a relative retention time of about 0.65 related to menadione sodium bisulfite.

Assessment:

The potential degradation product (impurity) of the menadione sodium bisulfite was observed under stress test with heat. The degradation impurity is well separated from the menadione sodium bisulfite and other active ingredients. Peak (2) is the menadione and peak (1) is due to its impurity.

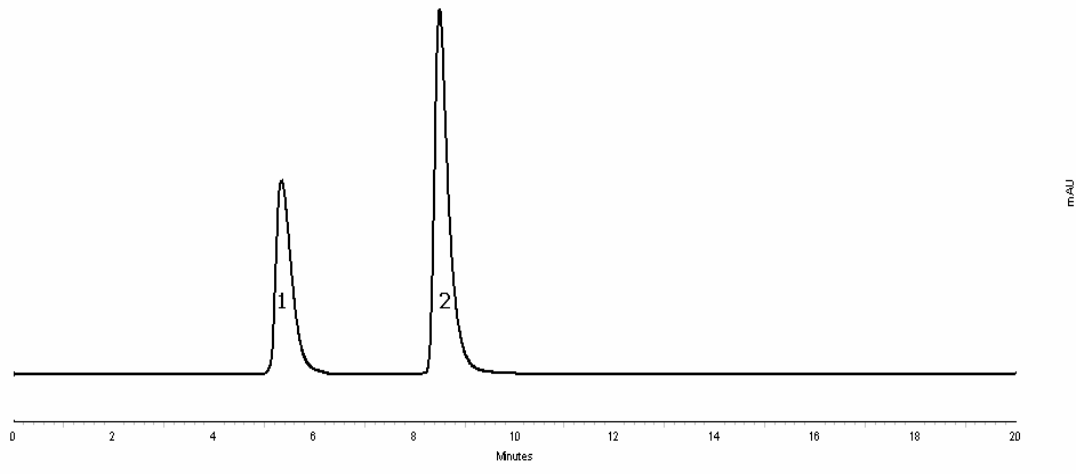


Figure (5.12): Stress test with heat for menadione sodium bisulfite.

5.7.2.2. Stress test under oxidation:

32 mg of menadione sodium bisulfite was mixed with 95 ml of 3% H_2O_2 for one hour and then diluted to 100 ml with 90% ACN. Finally, 5 ml of this solution is diluted to 50 ml with mobile phase.

Figure 5.13 illustrates clearly that menadione sodium bisulfite under stress conditions with 3% H_2O_2 did not show any potential degradation impurity. Peak (2) with relative retention time of 1.2 is due to H_2O_2 (was observed in the placebo).

Assessment:

No potential degradation products (impurities) of the menadione sodium bisulfite were observed under oxidation stress test. Peak (1) is the menadione and peak (2) is due to H_2O_2 in placebo.

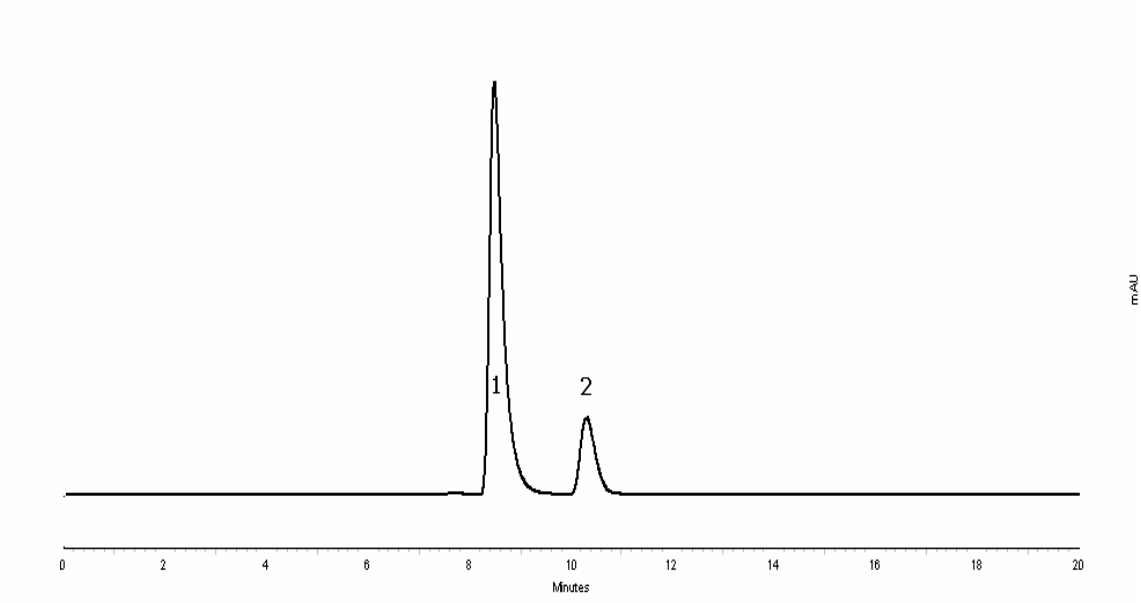


Figure (5.13): Stress test under oxidation for menadione sodium bisulfite

5.7.2.3. Stress test with light:

32 mg of menadione sodium bisulfite was stressed under day light for 72 hours and then dissolved in 100 ml 90% ACN. Finally, 5 ml of this solution is diluted to 50 ml with mobile phase.

Figure 5.14 illustrates clearly that menadione sodium bisulfite under light stress conditions shows an increase on unknown potential degradation impurity with relative retention time of about 0.65 related to menadione sodium bisulfite.

Assessment:

The potential degradation product (impurity) of the menadione sodium bisulfite was observed under stress test with light. The degradation impurity is well separated from the menadione sodium bisulfite and other active ingredients.

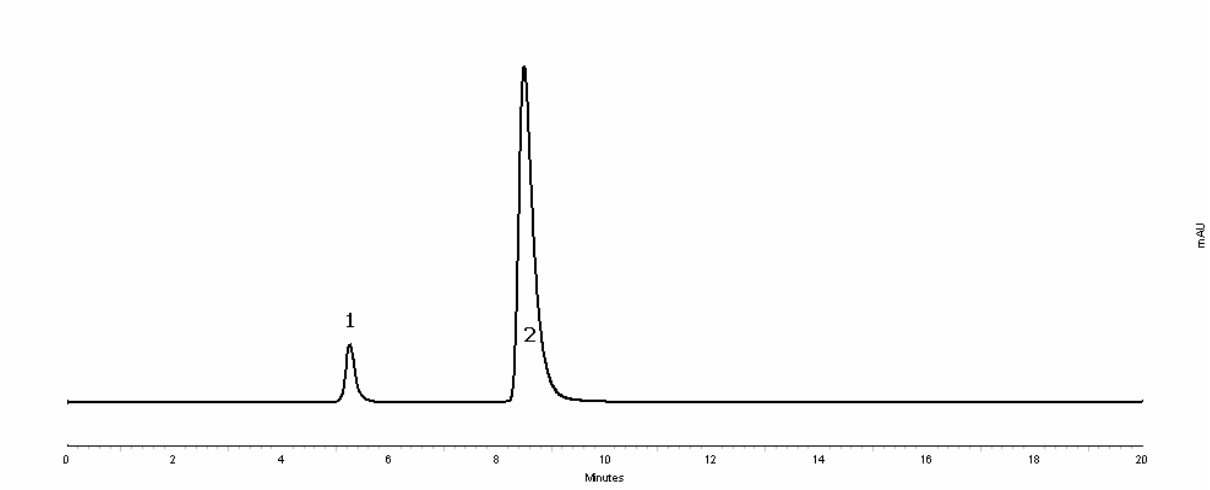


Figure (5.14): Stress test with light for menadione sodium bisulfite

5.7.2.4. Stress test with hydrochloric acid:

32 mg of menadione sodium bisulfite was mixed with 20 ml of 1 M HCl for 24 hours, neutralized with 1M NaOH and then diluted to 100 ml with 90% ACN. Finally, 5 ml of this solution is diluted to 50 ml with mobile phase.

Figure 5.15 illustrates clearly that menadione sodium bisulfite under stress conditions with HCl shows a slight increase on unknown potential degradation impurity with a relative retention time of about 0.65 related to menadione sodium bisulfite.

Assessment:

The potential degradation product (impurity) of the Menadione sodium bisulfite was observed under stress test with HCl. The degradation impurity is well separated from the menadione sodium bisulfite and other active ingredients. Peak (2) is the menadione and peak (1) is due to its impurity.

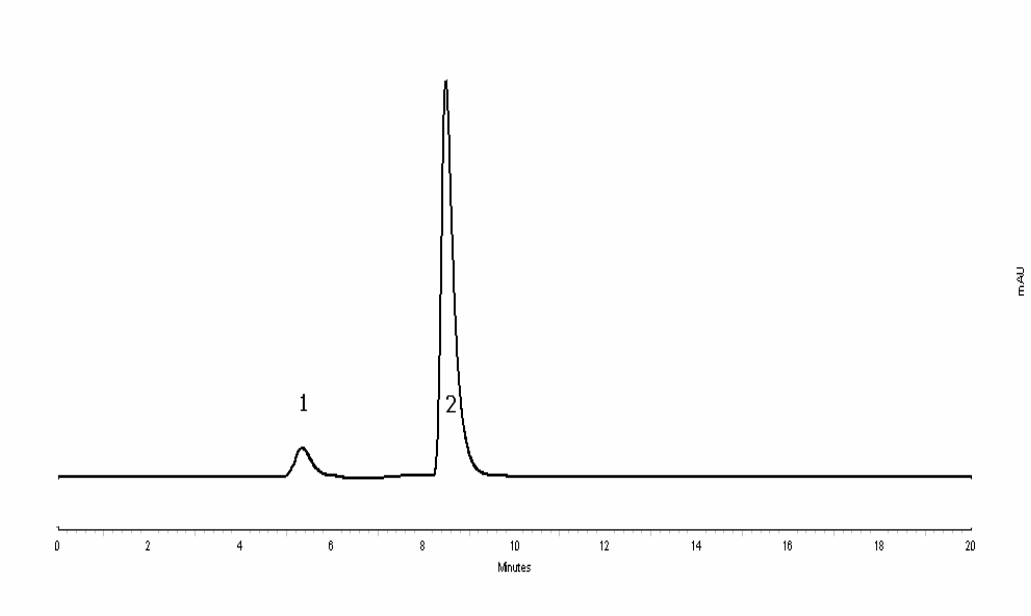


Figure (5.15): Stress test with hydrochloric acid for menadione sodium bisulfite.

5.7.2.5. Stress test with Sodium Hydroxide:

32 mg of menadione sodium bisulfite was mixed with 20 ml of 1 M NaOH for 24 hours, neutralized with 1M HCl and then diluted to 100 ml with 90% ACN. Finally, 5 ml of this solution is diluted to 50 ml with mobile phase.

Figure 5.16 illustrates clearly that menadione sodium bisulfite under stress conditions with sodium hydroxide shows an increase of five unknown potential degradation impurities with relative retention times of about 0.65, 0.82, 0.87, 0.92, and 1.2 related to menadione sodium bisulfite. It was clear that NaOH destroyed the compound completely and no menadione peak was found.

Assessment:

The potential degradation products (impurities) of the menadione sodium bisulfite were observed under stress test with NaOH. The unknown degradation impurities are well separated from the menadione sodium bisulfite and other active ingredients.

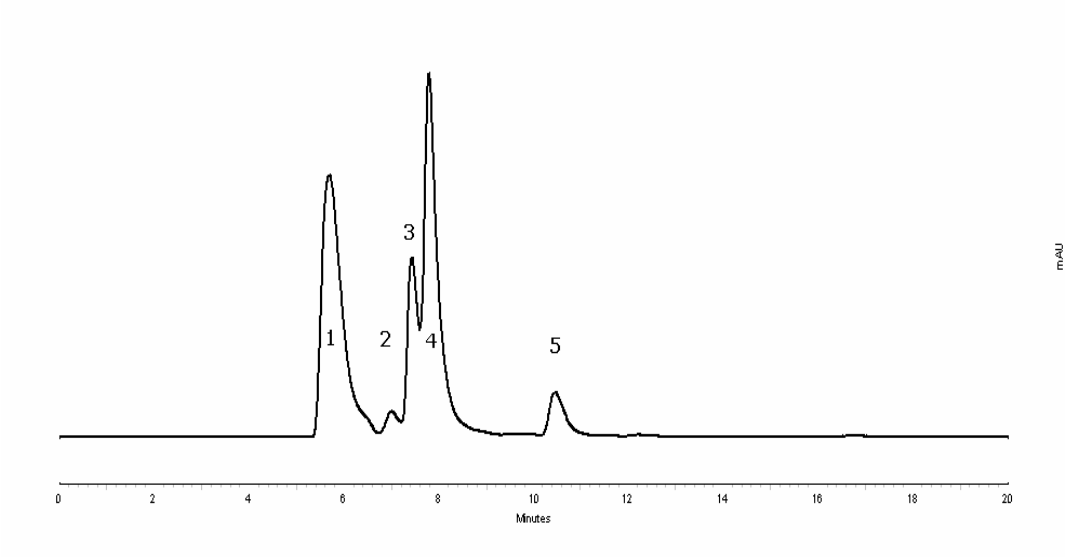


Figure (5.16): Stress test with sodium hydroxide for menadione sodium bisulfite

5.7.4. Stress tests for Amprolium hydrochloride:

5.7.4.1. Stress test with heat (105°C):

20 mg of amprolium hydrochloride was heated for 72 hours at 105°C in an oven and then dissolved in 100 ml 90% ACN. Finally, 5 ml of this solution is diluted to 50 ml with mobile phase.

Figure 5.17 illustrates clearly that amprolium hydrochloride under heat stress conditions shows a slight increase on unknown potential degradation impurity with relative retention time of about 0.3 related to amprolium hydrochloride.

Assessment:

The potential degradation product (impurity) of the amprolium hydrochloride was observed under stress test with heat. The degradation impurity is well separated from the amprolium hydrochloride and other active ingredients. Peak (2) is the amprolium HCl and peak (1) is due to its impurity.

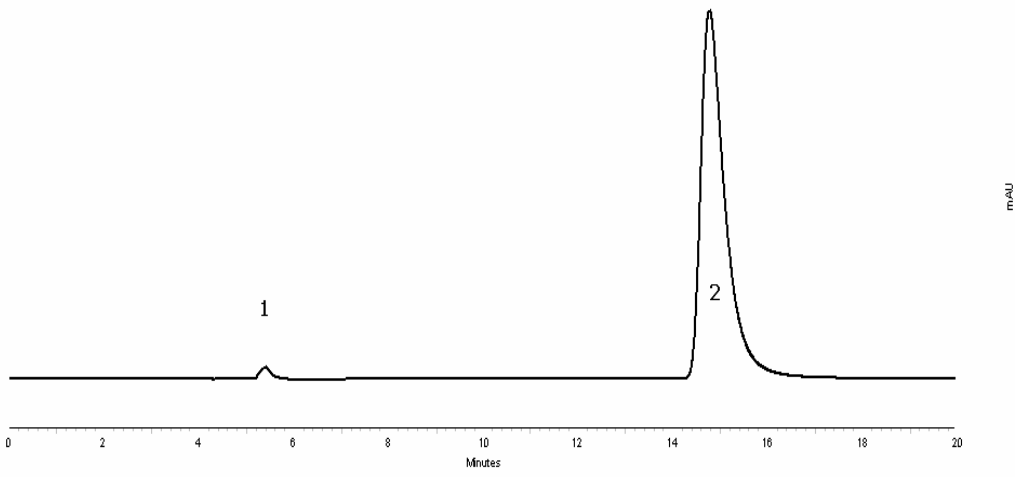


Figure (5.17): Stress test with heat for amprolium hydrochloride.

5.7.4.2. Stress test under oxidation:

20 mg of amprolium hydrochloride was mixed with 95 ml of 3% H_2O_2 for one hour and then diluted to 100 ml 90% with ACN. Finally, 5 ml of this solution is diluted to 50 ml with mobile phase.

Figure 5.18 illustrates clearly that amprolium hydrochloride under stress conditions with 3% H_2O_2 did not show any potential degradation impurity. Peak (1) with relative retention time of 0.70 is due to H_2O_2 in placebo.

Assessment:

No potential degradation products (impurities) of the amprolium hydrochloride were observed under stress test with oxidation. Peak (2) is the amprolium hydrochloride and peak (1) is due to H_2O_2 in placebo.

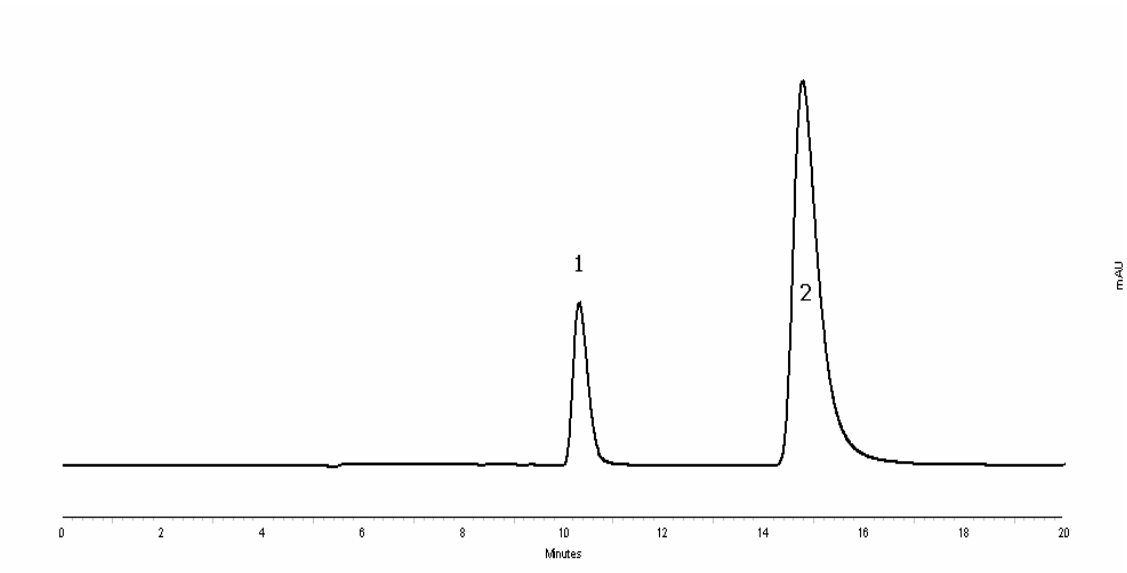


Figure (5.18): Stress test under oxidation for amprolium hydrochloride.

5.7.4.3. Stress test with light:

20 mg of amprolium hydrochloride was stressed under day light for 72 hours and then dissolved in 100 ml 90% ACN. Finally, 5 ml of this solution is diluted to 50 ml with mobile phase.

Figure 5.19 illustrates clearly that amprolium hydrochloride under light stress conditions shows a slight increase in an unknown potential degradation impurity with relative retention time of about 0.32 related to amprolium hydrochloride.

Assessment:

The potential degradation product (impurity) of the amprolium hydrochloride was observed under stress test with light. The degradation impurity is well separated from the amprolium hydrochloride and other active ingredients. Peak (2) is the amprolium HCl and peak (1) is due to its impurity.

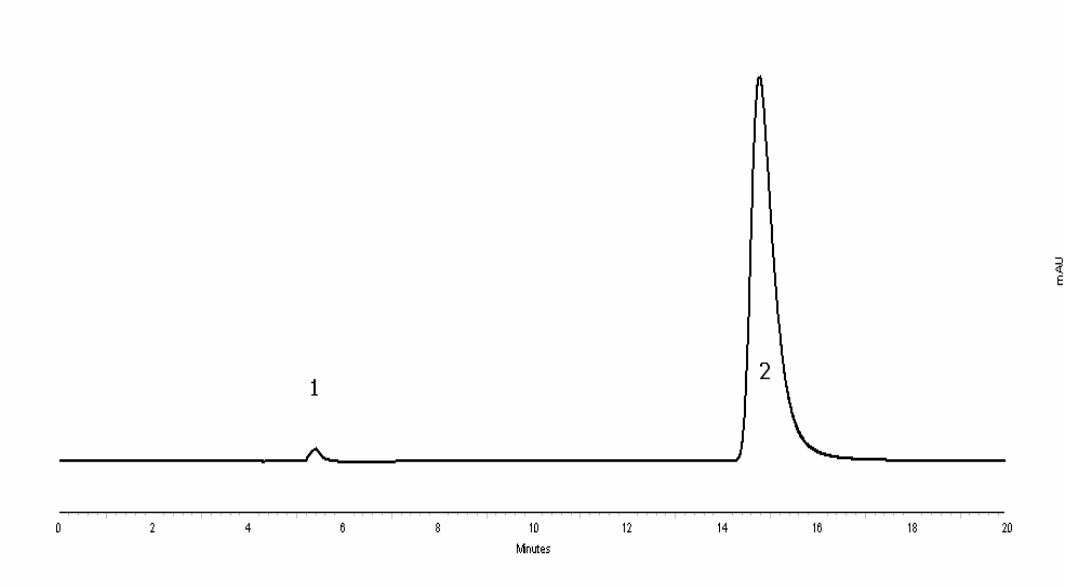


Figure (5.19): Stress test with light for amprolium hydrochloride.

5.7.4.4. Stress test with hydrochloric acid:

20 mg of amprolium hydrochloride was mixed with 20 ml of 1 M HCl for 24 hours, neutralized with 1M NaOH and then diluted to 100 ml with 90% ACN. Finally, 5 ml of this solution is diluted to 50 ml with mobile phase.

Figure 5.20 illustrates clearly that amprolium hydrochloride under stress conditions with HCl shows a slight increase on unknown potential degradation impurity with a relative retention time of about 0.37 related to amprolium hydrochloride

Assessment:

The potential degradation product (impurity) of the amprolium hydrochloride was observed under stress test with HCl. The degradation impurity is well separated from the amprolium hydrochloride and other active ingredients. Peak (2) is the amprolium HCl and peak (1) is due to its impurity.

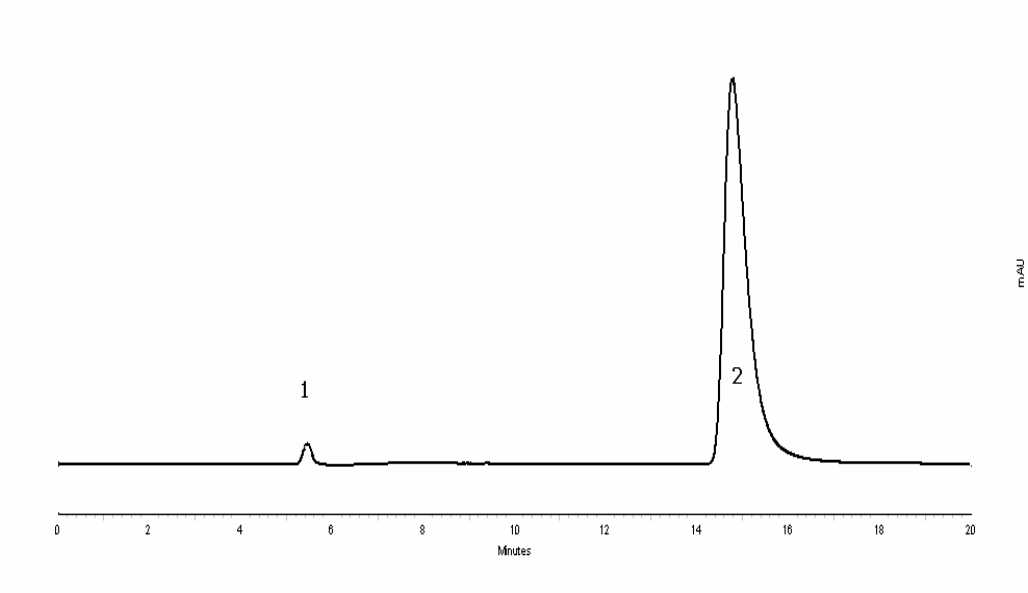


Figure (5.20): Stress test with hydrochloric acid for amprolium hydrochloride.

5.7.4.5. Stress test with Sodium Hydroxide:

20 mg of amprolium hydrochloride was mixed with 20 ml of 1 M NaOH for 24 hours, neutralized with 1M HCl and then diluted to 100 ml with 90% ACN. Finally, 5 ml of this solution is diluted to 50 ml with mobile phase.

Figure 5.21 illustrates clearly that amprolium hydrochloride under stress conditions with sodium hydroxide shows an increase on six unknown potential degradation impurities with relative retention times of about 0.38, 0.45, 0.50, 0.58, 0.69 and 0.94 related to amprolium hydrochloride.

Assessment:

The potential degradation products (impurities) of the Amprolium hydrochloride were observed under stress test with NaOH. The unknown degradation impurities are well separated from the amprolium hydrochloride. However, there was an overlap between one of the degradation impurities and menadione. This seems to be inevitable since the NaOH completely destroyed the compound. No peak for amprolium HCl could be found.

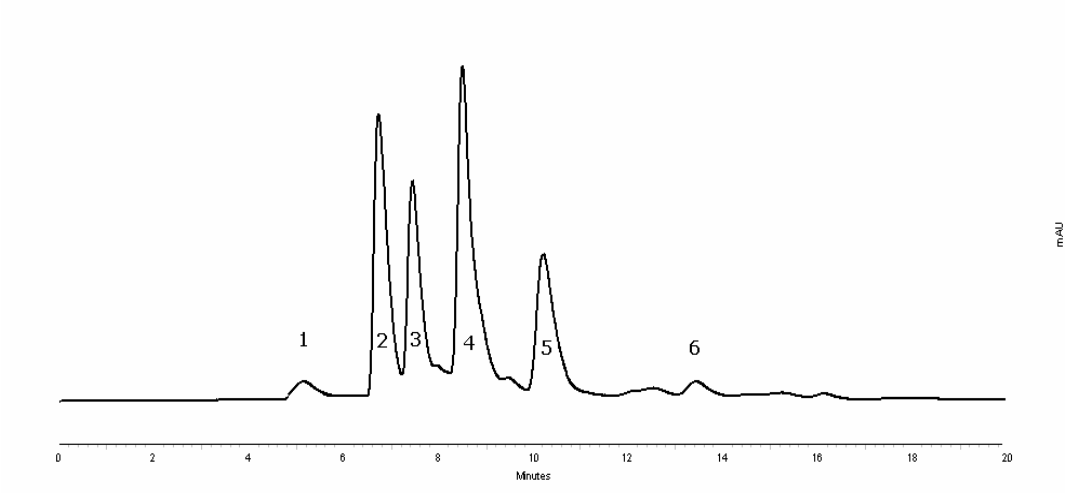


Figure (5.21): Stress test with sodium hydroxide for Amprolium hydrochloride.

In case of amprolium hydrochloride degradation under sodium hydroxide, the degradation pathway is complex and the decomposition products may be only observed under forcing

conditions and are unlikely to be formed under accelerated or long term testing. Therefore it may not be necessary to examine specifically these degradation products since they are not formed in practice as we have seen upon carrying the test of accelerated testing at 40°C and 75% relative humidity for six months as recommended for the A.S.K powder product (i.e. sealed aluminum package).We did not observe any degradation for amprolium injected separately. [58]

Chapter Six

Conclusion and Recommendations:

The proposed optimized HPLC method described herein was evaluated over the linearity, precision, accuracy, specificity, ruggedness and robustness. The method proved to be convenient and effective for the quality control of sulfaquinoxaline sodium, vitamin K_3 sodium bisulfite, and amprolium hydrochloride in A.S.K powder. It does not suffer any positive or negative interference due to common excipient present in the formulation and can be conveniently used for routine quality control analysis. All the assay validation results were within the allowed specifications of ICH/USP guidelines. The proposed optimized method is rapid, selective, requires a simple sample preparation procedure, and represents a good procedure for the simultaneous determination of the combined drugs in A.S.K formulation.

Moreover, this method is capable of distinguishing between the compounds of interest and their main degrades, which are expected to be present in the product since it passes the test of stability under stress conditions.

A.S.K Powder is difficult to separate via typical reverse phase HPLC or ion-pair RP-HPLC, but is readily separated by the ZIC®-HILIC column. This method is very sensitive and the assay can be performed only within 18 minutes.

Because of its novel column chemistry and unique selectivity, the ZIC-HILIC column allows excellent separation with faster analysis time, less organic solvent consumption and with no ion-pair reagent usage which make the routine analysis much cheaper. Reversal of elution order was noticed in comparison to RPLC mode.

Good linearity was shown over the 12–26 µg/ml levels for sulfaquinoxaline sodium and amproliun HCl and over the 0.12–0.26 µg/ml levels for menadione Recovery was found to be within the acceptable criteria (96.0- 104.0%) for all compounds of interest along the linearity range.

Recommendations:

1.) As seen some components of the product is affected greatly with heat especially menadione so the prepared solution is best to be kept in refrigerator in a closed container if need to be used for long time or prepared fleshly or either to be kept at 15°C for 24 hours as shown in solution stability.
2.) The product must be protected form sodium hydroxide since it destroy ed completely some of the product components.
3.) The assay needs to be used to compare the innovative product to the one from Pharmacare in order to establish similarity required for the product registration
4.) Specifications need to be set for the product to pass
5.) The assay may be used to determine the best conditions for the farmers to prepare the formulation. For example, the introduction mentioned that they prepare in water and dose for three days followed by an additional 3 days with a break in between. Can a farmer prepare one batch and use it for the whole treatment period or is it better to prepare for each day. This will depend on the stability.

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العنوان: تطوير طريقة جديدة وفعاله لتحليل مركبات السلفاكوينولين صوديوم ، وفيتامين ك٣ صوديوم ثنائي السلفايت ، والامبروليوم هيدروكلورايد مجتمعة معا في المستحضر البيطري A.S.K والمصنع في شركة دار الشفاء

إعداد الطالب: مشهور محمد فريد إسماعيل غانم.

إشراف الدكتور : صالح أبو لافي.

ملخص:

يتناول هذا البحث تقديم طريقة جديدة لتحليل مركبات السلفاكوينولين صوديوم (١)، وفيتامين ك٣ صوديوم ثنائي السلفايت (٢)، والامبروليوم هيدروكلورايد (٣) في المستحضر البيطري A.S.K الموجود على شكل بودرة.

لقد تمت عملية الفصل باستخدام الكروماتوغرافيا السائلة وقد استخدم العمود الكروماتوغرافي (ZIC-HILIC). أما الطور المتحرك فكان عبارة عن ٠,٢ مول/لتر محلول منظم من اسيتات الامونيوم مذاب في ماء نقي واسيتونيتريل (١٥٠ مل:٨٥٠ مل)، وقد ضبطت درجة الحموضة pH إلى القيمة ٥,٧ باستخدام حمض الخليك المركز وقد ضبطت طول الموجة عند ٢٦٣ نانوميتر.

لقد تم خلال هذا البحث دراسة الظروف العمليةالفضلى مثل تركيز الاسيتونيريل، تركيز اسيتات الامونيوم، درجة الحموضة للطور المتحرك على عملية الفصل للمركبات الثلاثة.

لقد تم إجراء دراسة التثبت لطريقة التحليل بعد تطويرها وقد وجد أن الطريقة قد اجتازت جميع الفحوصات المطلوبة حسب قوانين دستور الأدوية الأمريكي USP و ال ICH وهذا يبرهن على أن الطريقة صالحة لتحليل المركبات الثلاثة مجتمعة معا والموجودة في مستحضر ال A.S.K.

تمتاز الطريقة المطورة بالسرعة و قلة التكلفة اللازمة لتحليل الدواء، كما تمتاز بالكفاءة العالية والانتقائية وعند تطبيق الطريقة الجديدة على مستحضر A.S.K المصنع من قبل شركة دار الشفاء للأدوية وجد أن كل المركبات الفعالة لا تتداخل مع بعضها أو مع السواغ (المواد المضافة).

هذا وسيتم تقديم طلب اعتماد لهذه الطريقة الالية الجديده المطورة من قبل وزارة الصحة الفلسطينية قريبا.