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Development of Spectrophotometric Methods for Aliskiren Determination in Pharmaceutical Dosage Form

A thesis submitted in partial fulfillment of the requirements for the degree of master in pharmaceutical sciences

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Dedication

To my parents,

To my family,

To every one supported me. . . .

Declaration

I certify that this thesis submitted for the degree of master, is the result of my own research, except where otherwise acknowledged and this thesis (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signed ……..

Mohammed B. M. Abu Iriban

Date …….

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Abstract

Development of spectrophotometric methods for aliskiren determination in pharmaceutical dosage form.

Two simple, accurate and precise spectrophotometric methods for the determination of Aliskiren (ALS) in pharmaceutical products were developed. The first method was based on the reaction of ALS with 1,2-naphthoquinone-4-sulfonate (NQS) in alkaline medium producing an orange-red colored product, which absorbs maximally at 500 nm. The second method was based on the reaction of ALS with ninhydrin (NIN) mixed with ascorbic acid as reducing agent in phosphate buffer pH 6.0 producing blue-violet colored product, which absorbs maximally at 569 nm. The experimental parameters for both methods were studied and optimized. The optimum conditions were 1 ml 0.5% NQS solution, 1ml 0.01 M NaOH solution, water as diluting solvent and 10 min reaction time maintained at room temperature for NQS method. While for NIN method, 1ml 1.2% NIN mixed with 0.1% ascorbic acid dissolved in 0.2 M phosphate buffer pH 6.0, water as diluting solvent and 20 min reaction time maintained at 90±3°C. Furthermore, order of addition, stability of products chromogen and the stoichiometry of reactions were studied. The stability of products chromogen were 2 and 1.5 hr, respectively. The ratio between ALS; NQS and ALS; NIN was 1:2. Beer's law was obeyed in the concentration range of 20-300 and 10-170 µg/ml with R^2 of 0.991 and 0.992 for NQS and NIN methods, respectively. The validity of the methods was assessed according to International Conference on Harmonization (ICH) guidelines. Regarding accuracy, recovery values for NQS and NIN methods were 99.17–100.65 ± 0.28–1.7% and 99.63–101.2 ± 0.25–1.27%, respectively. The RSD for intra- and inter-assay precisions for NQS and NIN methods did not exceed 0.62 and 1.7% as well as 1.6% and 1.46%, respectively. Interferences liabilities were carried out to explore the effects of reagents and inactive ingredients. The average recovery values for 50 and 100 mg of ALS were 99.95 ± 1.8% and 100.86 ± 1% as well as 99.13 ± 0.76% and 100.3 ± 0.82% for NQS and NIN methods, respectively. The influence of small variation in the methods variables did not significantly affect the procedures; recovery values for NQS and NIN methods were 98.2–102.78 ± 0.14–1.6% and 98.6–101.1 ± 0.31–1.12%, respectively. The pharmaceutical dosage form was subjected for analysis of ALS content by developed methods and a reference one. The results were compared by statistical analysis with respect to accuracy and precision and no significant differences were found. The developed methods are easy to use, accurate and highly cost-effective for

routine analysis of ALS in quality control laboratories relative to HPLC and other techniques.

Keywords: Aliskiren, NQS, ninhydrin, spectrophotometric, validation.

ملخص الدراسة

تطوير طرق التحليل الطيفي للألسكرين في المستحضرات الصيدلانية.

يتضمن البحث تطوير طريقتين للتحليل الطيفي تتميز بأنها بسيطة ودقيقة لتقدير الألسكرين (ALS) في المستحضرات الصيدلانية. اعتمدت الطريقة الأولي علي تفاعل ALS مع 2,1- نافثوكينون سلفونات الصوديوم (NQS) في وسط قاعدي منتجة مركب ذو لون أحمر برتقالي وله أعلي شدة امتصاص عند 500 نانومتر. أما الطريقة الثانية اعتمدت علي تفاعل ALS مع النينهيدرين (NIN) في وجود حمض الأسكوربيك كعامل مختزل في محلول الفوسفات ذو درجة حموضة 6, لينتج مركب ذو لون أزرق بنفسجي له أعلي شدة امتصاص عند 569 نانومتر. وقد تم دراسة وتحديد ظروف التفاعل المثلي للطريقتين. الطريقة الأولي كانت أفضل الظروف عند استخدام 1مل, 0.5% NQS , 1مل, 0.01 مولاري هيدروكسيد الصوديوم, الماء في التخفيف, مدة التفاعل عشر دقائق في درجة حرارة الغرفة. أما الطريقة الثانية كانت أفضل الظروف 1مل, 1.2% NIN , مضاف اليه 0.1% حمض الأسكوربيك مذابين في محلول الفوسفات ذو درجة حموضة 6, والماء في التخفيف, مدة التفاعل عشرون دقيقة عند درجة حرارة 90±3 درجة مئوية. بالإضافة الي دراسة تأثير الترتيب المتبع في إضافة المتفاعلات, مدي استقرار الناتج ونسبة المتفاعلات في تكوين الناتج لكل طريقة. أظهرت النتائج استقرار الناتج حتي ساعتين, وساعة ونصف للطريقة الاولي والثانية علي الترتيب. وأن نسبة التفاعل في تكوين الناتج هي واحد ALS الي إثنين لكل من NQS وNIN. عند تقييم الجودة لطرق التحليل, وجد أنها تتبع قانون بير في مدي التركيز 20- 300 و10-170 ميكروجرام لكل مليلتر مع معامل ارتباط 0.991 و 0.992 للطريقة الأولي والثانية علي الترتيب. تم تقييم طريقتي التحليل حسب قواعد (ICH) تحت الظروف المثلي للتفاعل. عند فحص الدقة (Accuracy) كان معدل نسبة الاسترجاع (%Recovery) 99.17-100.65 ± 0.28-1.7% للطريقة الأولي, بينما 99.63-101.2 ± 0.25-1.27% للطريقة الثانية. عند اختبار الانحراف نتيجة تكرار طريقة التحليل في اليوم الواحد (Intra-assay) والتكرار من يوم لآخر (Inter-assay) لم يتجاوز الانحراف القياسي النسبي (%RSD) 0.62 و 1.7% للطريقة الأولي, و 1.6و 1.46% للطريقة الثانية علي الترتيب. عند دراسة تأثير المواد المتداخلة في طرق التحليل بالإضافة الي المكونات الغير أساسية في المستحضرات, وجد أن معدل نسبة الاسترجاع (%Recovery) عند خلط 50, 100 مليجرام من ALS بهذه الموادالمتداخلة كانت 99.95 ± 1.8% و 100.86 ± 1% للطريقة الأولي علي الترتيب, وكانت 99.13 ± 0.76% و 100.3 ± 0.82% للطريقة الثانية علي الترتيب. عند دراسة تأثير التغيرات الطفيفة في ظروف التفاعل علي نتائج طريقة التحليل, وجد أن معدل الاسترجاع (%Recovery) كانت 98.2-102.78 ± 0.14-1.6% و 98.6-101.1 ± 0.31-1.12% للطريقة الأولي والثانية علي الترتيب. تم استخدام طريقتي التحليل الطيفي المطورة بكفاءة في تحليل الألسكرين في المستحضرات الصيدلانية وقد قورنت بأحد النتائج المنشورة, وأظهر التحليل الإحصائي بأن الطرق كانت متماثلة. التحليل الطيفي للألسكرين باستخدام NQS وNIN تتميز بأنها بسيطة ودقيقة وغير مكلفة لعملية التحليل التقليدية في مختبرات الجودة مقارنة بالتحليل الكروماتوجرافي والطرق الأخري.

الكلمات المفتاحية: الألسكرين, 2,1- نافثوكينون سلفونات الصوديوم, النينهيدرين, التحليل الطيفي, تقييم الجودة.

Abbreviations

ACE-I	Angiotensin-converting enzyme inhibitors
ACPase	Carboxypeptidase
ALS	Aliskiren
ARBs	Angiotensin receptor blockers
AUC	Area under the curve
C_{max}	Maximum plasma concentration
DMF	*N*,*N'*-dimethyl formamide
DYDA	Diketohydrindylidene-diketohydrindamine
FDA	Food and Drug administration
ICH	International Conference on Harmonization
LOD	Limit of detection
LOQ	Limit of quantitation
MEKC	Micellar electrokinetic chromatography
µg	Microgram
ml	Milliliter
Nm	Nanometer
NIN	Ninhydrin
NQS	1,2-Naphthoquinone-4-sulfonate
PDA	Photodiode array
RAAS	Renin-angiotensin-aldosterone system
RSD	Relative standard deviation
TFA	Trifluoroacetic acid
US	United States
UV	Ultraviolet
UV/Vis	Ultraviolet/Visible
λ_{max}	Maximum wavelength
ε	Molar absorptivity

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Chapter 1

INTRODUCTION

1.1 Pharmaceutical analysis and quality control

Drug analysis is mainly divided into three fields; pure active ingredients (active ingredient analysis), pharmaceutical dosage forms (tablets, ointments, tinctures, suppositories, infusion, drops, etc), which consists of pharmaceutically active substance and at least one pharmaceutical excipient and bioanalysis of samples obtained *in vitro* and *in vivo* (serum, blood, urine, etc). The former two are controlled by regulations such as pharmacopoeias, International Conference on Harmonisation of Technical Requirements for Registration of pharmaceuticals for Human Use guidelines (ICH) and the physico-chemical properties of drugs summarized partially in book series entitled (Analytical Profiles of Drug Substances and Excipients), and are considerably less complex than methods for biological analysis. However, the analysis of a drug in pharmaceutical formulations is as important as bioanalysis, because the pharmaceutical product quality is directly related to the patient health (*Toyo'oka, 1999*).

Pharmaceutical analysis provides information on the identity, purity, content and stability of starting materials, active pharmaceutical ingredients and excipients. In details, we can know the identity of a drug in a formulated product, purity of a pure drug substance and excipients, the percentage of a stated content of a drug present in a formulation, if a formulation contain only the active ingredient or additional impurities are present and determine it's concentration, and the stability of a drug in a formulation. Moreover, we can determine the partition coefficients, pka value(s), solubilities and stability of a drug substance under development. Furthermore, a concentration of a drug in a sample of tissue or biological fluids can also be determined (*Watson, 2005*).

In the drug development and pharmaceutical control, chemical analysis plays a key role to ensure a high efficacy and safety for patients. For that, appropriate methods of quality control (qualitative and quantitative analyses, purity testing, chiral separation and related substance) are of so importance to the pharmaceutical industry (*Bonfilio et al, 2010* and *Suntornsuk, 2010*).

Analytical method development and validation play a major role in the discovery, development, and manufacture of pharmaceuticals. The validation of analytical method is used to demonstrate that the method is fitted for its purpose, so analytical methods need to be validated or revalidated prior to their introduction into routine analyses. The validation

procedure of the methods are carried out according to the ICH guidelines by determining the following parameters: specificity, linearity, range, precision (repeatability, intermediate precision and reproducibility), accuracy, limit of detection (LOD), limit of quantitation (LOQ), and robustness (*ICH, 2005*).

Thus, the pharmaceutical quality control should ensure use of appropriate analytical methods, of which it is observed a trend to utilize faster and more efficient techniques with cost savings and minimize solvent consumption (*Bonfilio et al, 2010*).

The quality of a product may deviate from the standard required, but in carrying out an analysis one also has to be certain that the quality of the analysis itself is of the standard required (*Watson, 2005*). The safety and efficacy of drug therapy can be ensured using a validated analytical method to assess the quality of pharmaceutical products as it has been considered suitable for their intended purpose.

1.2 UV/Vis spectrophotometry and derivatization

Monographs of pharmacopoeia contain tests on active ingredients, impurities and on different dosage forms contain one active ingredient or mixture. Highly developed, expensive instrumental techniques are used for this purpose (*USP, 2003 and BP, 2003*). On the other hand, pharmacopoeial methods still rely heavily on simple analysis by UV/Vis spectrophotometry (*Watson, 2005*).

The UV/Vis spectroscopy is very useful in quality control of pharmaceutical products due to the potential of the great majority of drugs to absorb energy in these wavelengths. This method is easy-to-use, inexpensive and robust. Offering good precision for quantitative measurements of drugs in formulations, also it's a routine method for determining some of the physico-chemical properties (partition coefficient, pKa, solubility, and release of a drug from a formulation) which need to be known for the purpose of formulation (*Watson, 2005*).

The limitation of this method is having moderate selectivity which depends on the chromophore of the individual drugs, so the drug which has extended chromophore as coloured drugs is more distinctive than a drug with a simple chromophore (*Watson, 2005 and Paim et al, 2008*).

Enhancement of sensitivity and selectivity in this method is achieved through several reactions with different reagents. This could be simply bringing about a shift in wavelength (*Nagaraju et al, 2008; Adegoke and Umoh, 2009; Bonfilio et al, 2010; Jadhav et al, 2010 and Reddy et al, 2011*).

Derivatization in analysis means basically the reaction caused by chemical reagents (chemical reactions) or physical methods to convert a poor detector-responding analyte into a highly detectable product, which make enhancement of properties as detector response, linear response range and the ability of separation (*Krull et al, 1994*). There are many variables that control reaction completeness, speed, and specificity, which include temperature, solvents, catalysts, and supports. Each of these variables can be optimized within a specific application and determined according to satisfactory maximum colour intensity and reproducible λ_{max} values (*Blau and King, 1978 and Knapp, 1979*).

Some of the most common derivatizing reagents used in UV absorbance detection are 1-fluoro-2,4-dinitrobenzene (Sanger reagent), Phenyl Isothiocyanate, 4-N,N-dimethylaminoazobenzene-4-isothiocyanate, 1,2-naphthoquinone-4-sulfonate (NQS), Benzoyl chloride, Dansyl chloride, Ninhydrin (NIN), Dinitrophenyl hydrazine, Hydroxylamine, p-aminobenzoic ethyl ester, and 2-halopyridinium salts (*Toyo'oka, 1999*). The selection of such reagents depends on specific functional group of the analyte.

1.3 Aliskiren

Aliskiren (ALS), (2(*S*),4(*S*),5(*S*),7(*S*)-*N*-(2-carbamoyl-2-methylpropyl)-5-amino-4-hydroxy-2,7-diisopropyl-8-[4-methoxy-3-(3-methoxypropoxy)phenyl]-octanamide hemifumarate) figure (1.1), is the first in a class of drugs called direct renin inhibitor and has been approved recently in 2007 for the treatment of essential hypertension (*Zhao et al, 2006; Lam and Choy, 2007; Huang et al, 2008 and Limoges et al, 2008*). This new antihypertensive agent with a different mechanism of action, which blocks renin system at its rate-limiting step by directly inhibiting the catalytic activity of renin, thereby reducing generation of angiotensin I and angiotensin II (*Daugherty, 2008 and Vaidyanathan et al, 2008*). ALS plays an important role in the management of hypertension by effectively control blood pressure, which is important for the management or prevention of cardio-vascular diseases and their complications (*Lam and Choy, 2007*).

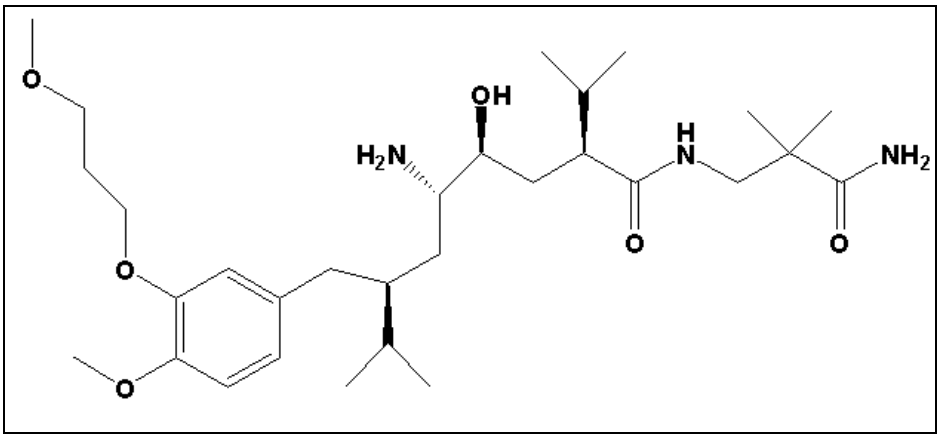

Figure 1.1: Chemical structure of aliskiren (free base).

Analysis of ALS was performed by different HPLC conditions mostly for determination of pharmacokinetic properties (*Lefevre and Gauron, 2000; Zhao et al, 2006; Vaidyanathan et al, 2007; Ayalasomayajula et al, 2008; Huang et al, 2008 and Limoges et al, 2008*). Few studies were published about ALS determination in dosage forms, plasma and urine (*Wrasse-Sangoi et al, 2010; Pachauri et al, 2010 and Babu et al, 2011*).

1.4 Justification

UV/Vis spectrophotometry is widely used for determination of pharmaceuticals (*Watson, 2005 and Paim et al, 2008*). In view of intrinsic characteristics as low cost, robust instrumentation, and procedures are generally simple, fast, accessible and well-performed without the use of organic solvents or hazardous chemicals which are detrimental to the environment, so this method is an ideal green analytical method suitable for the quality control of pharmaceuticals.

However, derivatization reactions are used in UV/Vis spectrophotometry to enhance sensitivity by improving the response of the analyte or to make a compound responsive which would not normally respond. Furthermore, to increase the detection response range of the analyte.

In this work, the sensitivity and detection response range of ALS will be enhanced by derivatization reactions with NQS and NIN. The formed products will shift the ALS spectrum to visible region, which is more accurate, specific and avoid interfering with any excipients or other ingredients.

1.5 Problem statement

ALS is a new drug approved in 2007, which until now has not official monographs in European, British and US pharmacopoeias. Furthermore, this is the first spectrophotometric study applied for ALS determination through derivatization reaction in dosage form.

Pharmaceutical counterfeiting is a worldwide public health problem, often under-recognized, especially in developing countries, where the percentage of counterfeit and sub-standard medicines is dramatically high (*Gaudiano et al, 2008*). For these reasons and novelty of the drug, the development of new sensitive, selective and simple technique is very important.

1.6 Aim

The aim of this study is to develop a sensitive spectrophotometric assay for ALS determination in pharmaceutical dosage form.

1.7 Objectives

- To develop spectrophotometric methods for assay of ALS through derivatization reactions with NQS and NIN.

- To optimize the conditions of derivatization reactions (pH, temperature, reagents concentration, solvents and time of the reaction), for both method.

- To determinate the stoichiometric ratio between ALS; NQS and ALS; NIN and postulated a pathway for the reactions.

- To validate the assay of ALS by determining the parameters (linearity, range, accuracy, specificity, precision, LOD, LOQ, robustness and ruggedness), for both method.

- To test possible interactions of pharmaceutical excipients with the developed methods.

- To apply the developed methods on pharmaceutical dosage form of ALS.

- To compare the developed methods with each other and with other published analytical method for ALS determination in dosage form.

Chapter 2

LITERATURE REVIEW

2.1 Spectrophotometry and derivatization

Analytical chemistry is described as the area of chemistry responsible for characterizing the composition of matter, both qualitatively and quantitatively. However, it is more than equilibrium chemistry and a collection of analytical methods; it is an approach to solving chemical problems. It has a wide applications in forensics, bioanalysis, clinical analysis, environmental analysis, quality control of industrial manufacturing and materials analysis. These fields need simple, rapid and accurate methods for routine analysis (*Harvey, 2000*). Analysts have developed lately an extensive array of instrumental techniques, which are extremely sensitive and can yield results rapidly to a high degree of accuracy. Among the instrumental analytical techniques, spectrophotometric technique occupies a unique position because of its simplicity, sensitivity, accuracy and rapidity. The availability of spectrophotometer made this technique indispensable to the modern analytical chemists (*Dehahay, 1967*).

Spectrophotometric methods for the determination of drugs are commonly based on the absorption of the visible and near ultraviolet radiation. Formerly, visible spectrophotometry was often called colorimetry and even now such definitions as colorimetric, photometric or absorptiometric methods are sometimes used in the literature, as equivalents to the term spectrophotometric method (*Gorog, 1995*).

The basis of spectrophotometric method is the simple relationship between the absorption of radiation by a solution and the concentration of species in the solution. There are only a few substances, which give sufficiently intense absorption and are spectrophotometrically measurable. Majority of species are generally determined indirectly by spectrophotometer in a variety of ways, such as

(i) Substances can be converted by a suitable reagent to an absorbing product changing oxidation state to a colored valence state.

(ii) Adding complexing agent to get colored complexes and so on.

(iii) Organic complexing agents are found to be more selective and sensitive color developing agents.

The criteria for a satisfactory colorimetric analysis are:

1. *Specificity of the color reaction*. Very few reactions are specific for a particular substance, but many give colors for a small group of related substances only, i.e. they are

selective. By utilizing such devices as the introduction of other complex-forming compounds, by altering the oxidation states, and control of pH, close approximation to specificity may often be obtained.

2. Proportionality between color and concentration. The color intensity increases with the increase in the concentration and it is desirable that the system obeys Beer's law.

3. Stability of the color. The color produced should be sufficiently stable to permit an accurate measurement to be taken. In other words, the period of maximum color must be long enough for precise measurements to be made. Moreover, the influence of other substances and experimental conditions (temperature, pH, stability in air, etc.) should be known.

4. Reproducibility. The colorimetric procedure must give reproducible results under specific experimental conditions.

5. Clarity of the solution. The solution must be free from precipitate if comparison is to be made with a clear standard. Turbidity scatters as well as absorbs the light.

6. High sensitivity. It is desirable, particularly when minute amounts of substances are to be determined, that the color reaction be highly sensitive. It is also desirable that the reaction product absorbs strongly in the visible rather than in the ultraviolet; the interfering effect of other substances in the ultraviolet is usually more pronounced (*Jeffery et al, 1989*).

Derivatization is a general term used for a chemical transformation designed to improve analytical capabilities. The chemical structure of the substance can be modified by the reaction of a specific functional group in the substance with derivatizing reagent resulting in a new chemical property that can be used for quantification or separation of the substance. Derivatization reactions can be induced by organic or electrochemical reactions, such as reduction or oxidation, or by displacement or addition reactions. Addition reaction result from an electrophilic or nucleophilic attack, which is determined by the reactivity of the substrate. Reduction and oxidation reactions are relatively non-specific and therefore less appropriate. Derivatization reactions are chemical reactions. As such, there are many variables that control reaction completeness, speed, and specificity. These include temperature, solvents, catalysts, and supports. Each of these variables can be optimized within a specific application (*Blau and King, 1978 and Knapp, 1979*).

A derivatization reaction is very often required in order to increase sensitivity or selectivity and can be achieved by a specific detection, such as fluorescence or absorption in the visible region. Derivatization reactions have many applications and particularly important

for trace analyses of complex biological samples and can be used for enantiomeric separation (*Toyo'oka, 1999*).

Derivatization reagents may be divided into four groups (*Laurence, 2000*).

1. Non-fluorescent reagents, generally used in UV/Vis (benzoyl chlorides, sulfonyl benzene and NIN).

2. Fluorogenic reagents, generally non-fluorescent, but which react with target compounds to form conjugated fluorescent cyclic molecules (fluorescamine and NQS).

3. Fluorescent reagents, which have a highly fluorescent aromatic group (fluorophore) and a reactive group.

4. Reagents with redox properties, used in electrochemistry.

2.2 1,2-Naphthaquinone-4-sulfonic acid sodium salt (NQS)

2.2.1 History

Folin's reagent or sodium NQS is a chemical reagent solid in state with orange color and has melting point about 289°C with decomposition, soluble in water and cold water. It is used to measure levels of amines and amino acids in a basic medium giving a derivative with a bright red color as shown in figure (2.1), which is detected at 305 and 480 nm (*Saurina and Hernandez-Cassou, 1993[a]; 1993[b] and 1994*). Folin firstly described a method for determining amino acids that depends on the combination of the amino group with sodium NQS in an alkaline solution to form colored compound (*Folin, 1922*).

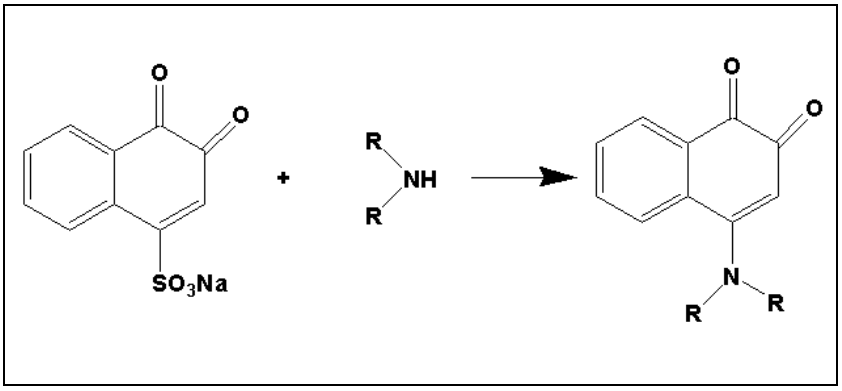

Figure 2.1: The reaction of sodium NQS with amines.

NQS has been used as derivatizing reagent in the development of both spectrophotometric and fluorimetric methods for determination of many pharmaceutical amines (*Li et al, 2007; Hasani et al, 2007 and Li and Yang, 2007*).

2.2.2 Optimization parameters for the reaction of NQS with amine group

2.2.2.1 NQS Concentration

Studying the effect of NQS concentration on its reaction with drugs that contain amino group revealed that the reaction was dependent on the NQS concentration as the absorbance increased with the increase in the reagent concentration (*Darwish et al, 2009; Aswani Kumar et al, 2010; 2011[a]; 2011[b]; Sowjanya et al, 2011 and Darwish et al, 2012*). These studies carried out the effect of NQS concentration by using reagent concentrations of 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, and 1%. Higher NQS concentration up to 1.25% had no effect on the absorption values.

2.2.2.2 Alkalinity and pH

Alkaline media is very necessary for the reaction of NQS with amino group, that to generate the nucleophile and activate nucleophilic substitution (*Darwish et al, 2009; Aswani Kumar et al, 2010; 2011[a]; 2011[b]; Sowjanya et al, 2011; Darwish et al, 2012 and Ali and Elbashir, 2012*). Different inorganic bases were tested: sodium hydroxide (NaOH), disodium hydrogen phosphate, and sodium carbonate, all prepared as aqueous solution of a concentration range of 0.0005-0.3 M (*Aswani Kumar et al, 2010 and 2011[b]*) and 0.01-0.05 M (*Aswani Kumar et al, 2011[a]*). The best results were obtained with NaOH in a concentration of 0.01 M solutions where with other bases either precipitation of white colloid occurred upon diluting the reaction solution with organic solvent, high blank readings, non reproducible results, and/or weak sensitivity were observed (*Aswani Kumar et al, 2010; 2011[a] and 2011[b]*).

The influence of pH on the reaction was also investigated by carrying out the reaction in buffer solution of varying pH values (*Darwish et al, 2009; Aswani Kumar et al, 2010; 2011[b] and Ali and Elbashir, 2012*). The results revealed that the absorbance at pH < 6 were close to 0, indicating that under acidic conditions the drugs have difficulty to react with NQS. This was probably due to the existence of the amino group of the drug in the form of hydrochloride salt, thus it loses its nucleophilic substitution capability. As the pH increased, the absorbance rapidly increased, as the amino group turns into the free amino group, which facilitating the nucleophilic substitution reaction. The maximum readings were attained in the range of pH at 10-11.5 (*Darwish et al, 2009; Aswani Kumar et al, 2010; 2011[b]*) and at pH of 12 (*Ali and Elbashir, 2012*). At higher pH values, sharp decrease in the absorption readings occurred. This was attributed probably to the increase in the amount of hydroxide ion that renders the reaction of amino group with NQS and the instability of NQS reagent (*Saurina and Hernandez-Cassou, 1993[a]*).

2.2.2.3 Diluting solvent

In order to select the most appropriate solvent for diluting the reaction solutions, different solvents were tested; as methanol, ethanol, isopropanol, acetone, acetonitrile, 1,4-dioxane, and water (*Darwish et al, 2009 and Darwish et al, 2012*). Colloids were obtained upon diluting the reaction solutions with water in the former study (*Darwish et al, 2009*), indicating the incomplete solubility of the product in water. In the second study (*Darwish et al, 2012*). Transparent solution was obtained upon diluting the reaction solutions with water, indicating the solubility of the product in water. However, other organic solvents were tested and compared with water, but the use of organic solvents leads to high analysis cost and more importantly, the incidence of exposure of the analysts to the side effects of these toxic solvents (*Fidler et al, 1987; Lindbohm et al, 1990; Wennborg et al, 2000; 2002 and Kristensen et al, 2008*). Therefore, water was used as a diluting solvent (*Darwish et al, 2012*).

In case of using inorganic bases in the reaction to make the alkaline media, best results were obtained when using NaOH and water as diluting solvent, where with other bases either precipitation of white colloid, high blank readings, non reproducible results, and/or weak sensitivity were observed upon diluting the reaction solution with organic solvent (*Aswani Kumar et al, 2011[a] and 2011[b]*).

2.2.2.4 Temperature and time of the reaction

The effect of temperature on the reaction were studied by carrying out the reaction at different temperatures (25-90° C) (*Darwish et al, 2009*), (25-80° C) (*Ali and Elbashir, 2012*) and (25-60° C) (*Darwish et al, 2012*). Some results revealed that there was no significant difference between the readings that have been obtained at room temperature and those at elevated temperatures (*Darwish et al, 2009 and Darwish et al, 2012*). Others revealed that the maximal absorbance was found at room temperature and at elevated temperatures the readings decreased rapidly (*Ali and Elbashir, 2012*).

The time that required for completion of the reaction was investigated by allowing the reaction to proceed at room temperature at different periods of time. It was found that the reaction goes to almost completion within 5 minutes (*Ali and Elbashir, 2012*). However, for higher precise readings, the reaction was allowed to proceed for longer quite time (10 minutes) (*Darwish et al, 2009 and Darwish et al, 2012*). Longer reaction time up to 25 minutes did not affect the reaction (*Darwish et al, 2009; Darwish et al, 2012 and Ali and Elbashir, 2012*).

2.2.2.5 Stability of product chromogen

The effect of time on the stability of the chromogen was studied by following the absorption intensity of the reaction solution (after dilution) at different time intervals. In several studies, it was found that the absorbance of the chromogen remained stable for at least 4 hours (*Darwish et al, 2009; Aswani Kumar et al, 2010; 2011[a] and 2011[b]*). In other studies, it remained stable for 1 hour (*Sowjanya et al, 2011 and Darwish et al, 2012*). This allowed the processing of large batches of samples with comfortable measurements and convenience.

2.2.3 Applications

NQS has been used as a chromogenic reagent for the spectrophotometric determination of many pharmaceutical amines. Some of recently published papers will be mentioned.

Aswani Kumar *et al.* have developed three specrtophotometric methods for determination of several pharmaceutical amines by derivatization reaction with NQS reagent (*Aswani Kumar et al, 2010; 2011[a] and 2011[b]*). These methods were based on the condensation of these drugs with NQS in alkaline media to yield orange colored products. The effective parameters on the reaction were studied, such as the effects of NQS concentration, alkalinity and pH. Furthermore, the effect of time on the stability of the chromogens was evaluated.

The following table (2.1) summaries the studied drugs, Beer's law limits, correlation coefficients and maximum wavelength for these methods.

Table 2.1: Summary of studied drugs in Aswani Kumar et al. studies.

Method	**Studied drugs**	**λ_{max}, nm**	**Beer's law limits (µg/ml)**	**Correlation coefficient (r^2)**	**Ref.**
1	Valacyclovir	495	20-120	0.9991	*Aswani Kumar et al, 2010*
	Cefotaxime	475	20-140	0.9996	
2	Cefadroxil	475	10-100	0.993	*Aswani Kumar et al, 2011[a]*
	Ceftriaxone	480	25-175	0.995	
3	Ceftazidime	495	20-80	0.9994	*Aswani Kumar et al, 2011[b]*
	Cefepime	475	20-140	0.998	

These methods were convenient with good validation results for assay of these drugs in their commercial pharmaceutical preparations.

Darwish *et al.* have developed two specrtophotometric methods for determination of fluoxetine hydrochloride and cinacalcet hydrochloride in their pharmaceutical dosage form through derivatization reaction with NQS. These methods were based on the reaction of fluoxetine and cinacalcet with NQS in alkaline media of pH 11 and pH 8.5 to form orange-red products that exhibiting maximum absorption at 490 nm with linearity in the concentration range of 0.3-6 µg/ml and 3-100 µg/ml and correlation coefficient of 0.9997 and 0.9993, respectively. Optimization for reaction variables was made by studying the effects of NQS concentration, pH, temperature, time of the reaction, diluting solvent and the stability of the chromogens. Both methods were validated and successfully applied to the determination of fluoxetine and cinacalcet in their pharmaceutical preparations (*Darwish et al, 2009 and Darwish et al, 2012*).

Sowjanya *et al.* have developed a spectrophotometric method for determination of pregabalin using NQS in bulk and pharmaceutical dosage form. This method was based on the condensation of pregabalin with 0.5% NQS in 0.01 M of NaOH solution to yield orange colored product, which absorbed maximally at 485 nm. Optimization of reaction parameters was evaluated by studying the concentration and volume of NQS and NaOH solution. Moreover, the stability of the chromogen was investigated. The linearity was observed in the concentration range of 5-45 µg/ml with correlation coefficient of 0.993. The reagents used were inexpensive, available and the procedures did not involve any critical reaction conditions or tedious sample preparation. This method was successfully applied for the assay of pregabalin in pharmaceutical preparations (*Sowjanya et al, 2011*).

Ali and Elbashir have developed a spectrophotometric method for determination of lisinopril dihydrate in pharmaceutical preparation using NQS as chromogenic reagent. This method was based on the formation of a purple compound measured maximally at 481 nm from the reaction of lisinopril and NQS in alkaline media of pH 12.0. The effects of NQS concentration, pH, temperature and standing time were studied and optimized. The linearity range was found to be 5-50 µg/ml with correlation coefficient of 0.9997. This method was rapid, simple, and economical. Moreover, it could be applied directly to the pharmaceutical sample without prior separation or treatment (*Ali and Elbashir, 2012*).

2.2.4 Toxicity

NQS may be irritating to eyes, respiratory system and skin. In case of contact with eyes or skin, rinse immediately with plenty of water and seek medical advice. Also, avoid

breathing dust, fume, gas, mist, vapors and spray *(Aldrich, 2012)*. Repeated or prolonged exposure is not known to aggravate medical condition and carcinogenic, mutagenic, teratogenic effects and development toxicity are not available.

2.3 Ninhydrin (NIN)

2.3.1 History

NIN was first prepared in 1910 by Siegfried Ruhemann while attempting to oxidize 1-hydrindone (1-indanone) to 1,2-diketohydrindene (1,2-indanedione) using *p*-nitrosodimethylaniline. However, instead of forming the expected product, two molecules of the nitroso compound had oxidized 1-hydrindone to produce the disubstituted hydrindone. The hydrindone product reacted rapidly with water to form the stable hydrate of triketohydrindene (ninhydrin) (*Ruhemann, 1910 and McCaldin, 1960*). It was significant that Ruhemann's observed the NIN reaction with ammonia under aqueous conditions to form a deep reddish-violet product which no longer had any reducing properties (*Ruhemann's* purple) (*Ruhemann, 1910*).

NIN (triketohydrindene hydrate) is a white to brownish white crystalline powder, soluble in water and alcohol. It turns red when heated above 100° C and its melting range is 240-245° C with decomposition.

NIN is extensively used in the analytical determination of amino acids and related structures (*Sheng et al, 1993 and Hsieh et al, 1995*). It can react also with a variety of primary and secondary amines producing *Ruhemann* purple color (*Wu et al, 2005; Siddiqui et al, 2010 and Nagaraja et al, 2011*).

When NIN reacts with alpha-amino acids, as shown in figure (2.2), carbon dioxide, and an aldehyde with one carbon less than the decarboxylated amino acid are produced. On heating the solution, the formed amine reacts with another NIN molecule to form a blue-to-purple color product with a maximum absorbance at 570 nm. Also, proline and hydroxyproline produce a yellow color when react with NIN under similar conditions with maximum absorbance at 440 nm (*Scott and Eagleson, 1988 and Stryer, 1988*). Moreover, NIN can react with a variety of primary and secondary amines under slightly acidic condition, resulting in a so-called *Ruhemann* purple color (diketohydrindylidene-diketohydrindamine, DYDA) (*Sheng et al, 1993*).

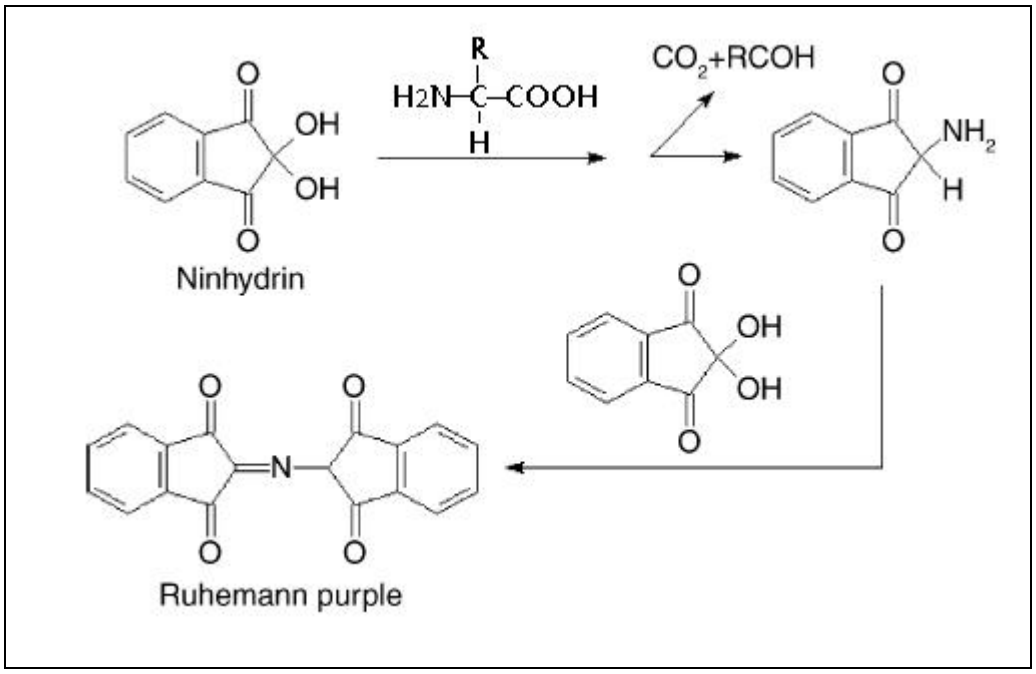

Figure 2.2: The reaction of ninhydrin with amino acid.

The most important factors involved in the quantitative production of *Ruhemann* purple include a high concentration of aqueous NIN solution, a high reaction temperature, and an optimum acidic condition (*Sheng et al, 1993; Curotto and Aros, 1993; Kim et al, 1994 Hsieh et al, 1995 and Wu et al, 2005*).

2.3.2 Optimization parameters for the reaction of NIN with amine group

2.3.2.1 NIN concentration

It is reported that, different concentrations of NIN, ranging from 0.05% to 5%, yielded *Ruhemann* purple of different intensity varying with different amines. Sheng, Kraft and Schuster mentioned that, when NIN concentration was increased gradually from 0.05% to 5% at a fixed concentration of asparagines, the color changed from light yellow to brownish purple (*Sheng et al, 1993*). Others also pointed out that; absorbance was increased with increases in NIN concentration from 0.2% to 1% (*Wu et al, 2005*). All samples showed purple colors which demonstrated maximum absorbance at 570nm.

2.3.2.2 Effect of pH

At high pH, the unprotonated amine group of the amino acids or the intermediate product can act as a good nucleophile, while at low pH these amine groups are protonated making them less nucleophilic, so that the reaction does not form the *Ruhemann's* purple product in acidic conditions (*Joullie et al, 1991*). The optimum pH for the reaction of NIN with amino acids is around pH 5; however this varies with the amino acid (*Moore and Stein, 1948*). Acid environment is one of the most important factors on the reaction of NIN with

amines (*Wu et al, 2005 and Nagaraja et al, 2011*). Nagaraja *et al.* investigated the effect of different pH values for several drugs and found that maximum color intensity was observed at pH 5.5 in citrate buffer for all the drugs studied (*Nagaraja et al, 2011*).

2.3.2.3 Reducing agents

The reaction of amino acids in acid solution with NIN to form the purple color is attributed to the anion of DYDA. It is believed that the first step in the reaction is the oxidative deamination of the amino acids with the formation of ammonia and the reduction of NIN to 2,2'-dihydroxy-[2,2'-bi-1H-indene]-1,1',3,3'-(2H,2H')-tetrone (hydrindantin). The ammonia then condenses with the hydrindantin to form DYDA (*Lamothe and McCormick, 1973*). Moore and Stein developed this reaction as a convenient photometric method for the determination of amino acids. Consistent and reproducible results were obtained by the introduction of stannous chloride, a reducing agent, into the system to prevent the oxidation of the hydrindantin (*Moore and Stein, 1948*). Yemm and Cocking used a solution of potassium cyanide as the reducing agent rather than the less stable stannous chloride (*Yemm and Cocking, 1955*). Ichishima also used this potassium cyanide ninhydrin reagent for the activity assay of acid carboxypeptidase (ACPase) from *Aspergillus* (*Ichishima, 1972*). However, the poisonous nature of potassium cyanide presents some problems regarding the routine assay of ACPase particularly for food companies. Yokoyama and Hiramatsu had investigated the direct use of ascorbic acid as a reducing agent instead of potassium cyanide for the photometric determination of amino acids (*Yokoyama and Hiramatsu, 2003*). Moreover, Moore and Stein used hydrindantin to prepare NIN reagent (*Moore and Stein, 1954*). However, the price of hydrindantin is about 100 times higher than that of ascorbic acid. Furthermore, the amount of hydrindantin required to prepare the same volume of NIN reagent was about 10-20 times larger than that of ascorbic acid (*Moore and Stein, 1954 and Ichishima, 2000*). Takahashi used sodium borohydride as a reducing agent for amino acid analysis (*Takahashi, 1978*). Unfortunately, sodium borohydride is difficult to handle for the preparation of NIN solution in standard laboratories due to its spontaneous combustion. Nagaraja *et al.* have developed a spectrophotometric method for the analysis of several drugs based on the reaction of these drugs with NIN derivatizing reagent and sodium molybdate as a reducing agent (*Nagaraja et al, 2011*). Also, several modifications have been proposed to enhance the sensitivity and application of NIN that include use of cadmium, iron, zinc, lithium and cyanide (*Friedman, 2004*).

2.3.2.4 Diluting solvent

NIN is a well-established reagent for the determination of certain amines, amino acids and thiophenes (*Figel, 1960*). The reaction is usually carried out by heating for a short time in an organic solvent (2-propanol, butanol, *N, N'*-dimethylformamide (DMF)) or in a mixture of water and organic solvent (*Gorog, 1995*). Krishna and Sankar have developed a spectrophotometric method for determination of alfuzosin via derivatization with NIN reagent in DMF solvent to produce a colored product which measured maximally at 575 nm without using any buffer systems (*Krishna and Sankar, 2007*). Moreover, Shiddiqui *et al.* have developed a spectrophotometric method for determination of gabapentin based on the reaction with NIN reagent in methanol, then diluted with deionized water, also without using any buffer systems. The maximum absorbance was measured at 568 nm (*Siddiqui et al, 2010*).

2.3.2.5 Temperature and time of the reaction

It is known that the reaction temperature is very important. Majority of the reported studies on NIN reactions have been performed at elevated temperature. In room temperature no color will be formed up to several hours, but when the reaction temperature increased, the time for the color formation was significantly decreased (*Friedman, 2004; Wu et al, 2005; Krishna and Sankar, 2007 and Nagaraja et al, 2011*).

2.3.2.6 Stability of product chromogen

The *Ruhemann's* purple product formed from the reaction of NIN with amino acids was not stable over long periods of time, but it was found that the stability could be improved by coupling it with a metal (*Kawerau and Wieland, 1951*). In several studies of pharmaceutical amines, the effect of time on the stability of colored product was studied. Nagaraja *et al.* demonstrated that the colored products for studied drugs which containing amine group were stable for at least 24 hours at room temperature (*Nagaraja et al, 2011*).

2.3.3 Applications

Reactions with NIN are widely used to analyze and characterize amino acids, peptides and proteins as well as numerous other NIN positive compounds in biomedical, clinical, food, forensic, histochemical, microbiological, nutritional and plant studies (*Friedman, 2004*). It has been extensively used in the determination of the compounds of pharmaceutical importance and in kinetic studies (*Rahman and Azmi, 2001 and Arayne et al, 2008*). Some of studies will be mentioned;

Nagaraja *et al.* have developed a spectrophotometric method for determination of isoniazid, lisinopril dihydrate, amoxicillin trihydrate, ampicillin trihydrate, glucosamine

sulfate, phenylpropanolamine hydrochloride and gabapentin in their pharmaceutical preparations (*Nagaraja et al, 2011*). The analysis was based on the reaction of drug molecules with NIN and sodium molybdate mixture in citrate buffer solution of pH 5.5 at 90±5 °C to give *Ruhemann's* purple with maximum absorbance at 570 nm. The reaction variables were studied and optimized; such as concentration of NIN and sodium molybdate mixture, pH, reaction time and temperature, as well as the stability of the colored products. The following table (2.2) summaries the studied drugs, Beer's law limits and correlation coefficients for this method.

Table 2.2: Summary of studied drugs in Nagaraja et al. study.

Studied drugs	**Beer's law limits (µg/ml)**	**Correlation coefficient (r)**
Isoniazid	0.5–5	0.9998
Lisinopril dihydrate	2.5–12.5	0.9999
Amoxicillin trihydrate	1.5–14	0.9996
Ampicillin trihydrate	3–20	0.9994
Glucosamine sulfate	1.2–7.5	0.9995
Phenylpropanolamine HCL	0.9–7	0.9999
Gabapentin	0.25–4.8	0.9997

This method was convenient with good validation results for assay of these drugs in their commercial pharmaceutical preparations.

Krishna and Sankar have developed a spectrophotometric method for determination of alfuzosin in bulk and tablets via derivatization with NIN (*Krishna and Sankar, 2007*). The method was based on the reaction of alfuzosin with NIN in DMF producing a colored product, which absorbed maximally at 575 nm. The experimental parameters were investigated; such as the volume of the reagent, selectivity of the solvent, reaction time and stability. Beer's law was obeyed in the concentration range of 12.5–62.5 µg/ml with correlation coefficient of 0.9999. The validity of the method was assessed and the method could be used in quality control laboratories for the determination of alfuzosin in pharmaceutical formulations.

Wu *et al.* have developed a method for determination of glucosamine release from sustained release hydrophilic matrix tablets via derivatization with NIN by spectrophotometer (*Wu et al, 2005*). The method was based on the reaction between NIN and glucosamine in phosphate buffer of pH 6.0 at 100 °C for 5 minutes to produce colored

product, which absorbed maximally at 570 nm. The optimization of the method was essential and carried out including the NIN concentration, reaction time, pH, reaction temperature and purple color stability period, as well as glucosamine/ninhydrin ratio. Glucosamine tablets (600 mg) with different hydrophilic polymers were formulated and manufactured on a rotary press. Dissolution studies were conducted according to USP using deionized water at 37±0.2 °C with paddle rotation of 50 rpm and samples were removed manually at appropriate time intervals. Glucosamine was quantitatively analyzed under optimized conditions and the calibration curve was constructed in the range of 0.202–2.020 mg with correlation coefficient of 0.9999. The method was easy to use, accurate and highly cost-effective for routine studies relative to HPLC and other techniques.

Siddiqui *et al.* have developed a spectrophotometric method for determination of gabapentin in pharmaceutical dosage forms. The method was based on the reaction of gabapentin with NIN in methanol heated on a water-bath at 70±5 °C for 15 minutes to give colored product, which was measured at 568 nm. The concentration of NIN, reaction time and temperature, as well as the effect of using different organic solvents as effective parameters on the reaction were studied. Linearity was obtained in the concentration range of 2–30 µg/ml with correlation coefficient of 0.9999. The method showed good validation results and had been successfully applied to the determination of gabapentin in pharmaceutical formulations (*Siddiqui et al, 2010*).

Frutos *et al.* have developed a colorimetric assay for gentamicin in poly-(methyl methacrylate) implant cements. The method was based on the reaction of NIN with primary and secondary amines present in gentamicin in phosphate buffer of pH 7.4 heated in a water bath at 95 °C for 5 minutes to produce purple color, which measured maximally at 400 nm. The effect of several factors including pH, NIN concentration and reaction time were investigated to optimize the assay method. The linearity concentration range was obtained at 30-120 µg/ml with correlation coefficient of 0.9996. The colorimetric assay of gentamicin was reproducible, sensitive, simple and inexpensive, as well as could be applied for routine analysis of gentamicin in quality control laboratories (*Frutos et al, 2000*).

2.3.4 Toxicity

Allergic contact dermatitis, immunoglobulin-E mediated rhinitis and occupational asthma were reported in a forensic technician (*Schlacke and Fuchs, 1989; Hytonen et al, 1996; Piirila et al, 1997; Murphy and Gawkrodger, 2000 and Soost et al, 2010*). Symptoms of

exposure to NIN may include a skin, eye and respiratory irritant. Moreover, may cause sensitization and redness of the skin. In case of contact with eyes or skin, rinse immediately with plenty of water and seek medical advice. Also, avoid breathing dust, fume, gas, mist, vapors and spray. In inhalation case, immediately leave the contaminated area and if symptoms develop (such as wheezing, cough, shortness of breath or burning in the mouth, throat or chest), call a physician and transport the victim to the nearest hospital (*Aldrich, 2012*).

2.4 Aliskiren

2.4.1 History

In the last thirty years ago, an intensive effort with several in vitro and in vivo studies has been focused on the development of effective and long-acting orally renin inhibitors (*Greenlee, 1990*). When the importance of renin and renin-angiotensin system in the control of fluid balance, hypertension and cardiovascular disease has become clear.

Renin inhibitors can be divided into three generations of compounds as illustrated in figure (2.3). First generation were discovered to block the enzymatic action of renin by peptide analogues of angiotensinogen and neither very potent, nor metabolically stable (*Szelke et al, 1982*). Second generation was dipeptide transition-state analogue inhibitors of the active site called peptidomimetic compounds (*Delabays et al, 1989; Boger et al, 1990; Clozel and Fischli, 1993 and Himmelmann et al, 1996*). This generation was potent (in nanomolar range) and lowered blood pressure in animals and in humans, when administered by a parenteral route. Their clinical uses were limited because of lack of oral activity and have short duration of action (*Weber et al, 1993 and Himmelmann et al, 1996*). Third generation, non-peptide-like compounds. The most successful one is aliskiren (*Rahuel et al, 2000 and Wood et al, 2003*). ALS is the first oral direct renin inhibitor approved by the United State of Food and Drug Administration (FDA) in March 2007 as mono or combination therapy with other antihypertensive agents to optimize blood pressure control. At this time, ALS should be considered as an alternative agent for mild-to-moderate hypertension or as an adjunctive therapy when preferred agents fail to maintain optimal blood pressure control (*Lam and Choy, 2007*).

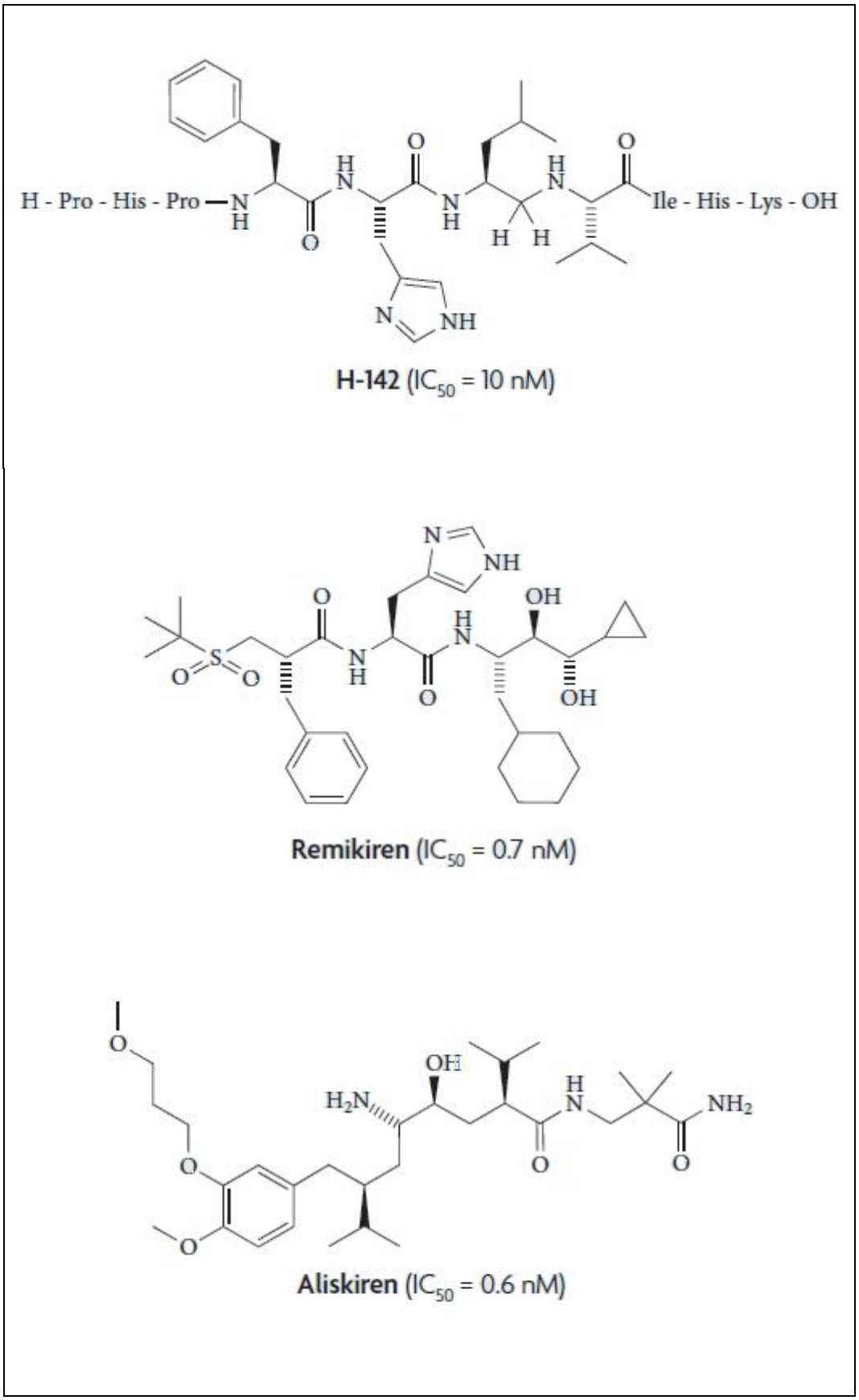

Figure 2.3: Chemical structures of three generations of renin inhibitors; the peptide analogue H-142, the peptidomimetic remikiren and the non-peptidic aliskiren.

2.4.2 Synthesis

The IUPAC name of ALS is, (2(*S*),4(*S*),5(*S*),7(*S*)-*N*-(2-carbamoyl-2-methylpropyl)-5-amino-4-hydroxy-2,7-diisopropyl-8-[4-methoxy-3-(3-methoxypropoxy)phenyl]-octanamide) figure (2.3). Due to four chiral centers of ALS, the initial synthesis of the compound was both difficult and expensive. This problem was solved through the development of a new and innovative synthetic route based on the synthon approach for commercial production (*Jensen et al, 2008*).

The key elements of this approach are the three different buildings blocks, synthon A, B and C figure (2.4), which can be synthesized independently. Synthon A can be produced by reactions involved a rhodium-catalyzed enantioselective hydrogenation. Synthon B obtained from a pig liver esterase-catalysed enzymatic resolution of a racemic ester and synthon C produced from a one-pot nitrile reduction/ester aminolysis.

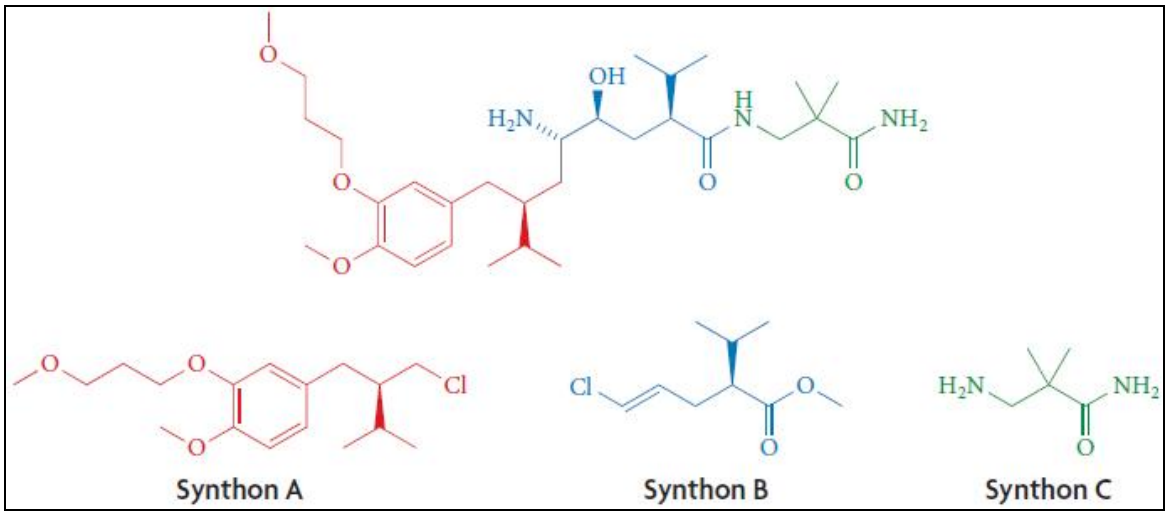

Figure 2.4: The synthon approach to produce aliskiren.

ALS synthesis consists of three sequence parts. First part, coupling of synthon A with synthon B by a nickel-catalysed cross-coupling reaction. Second part, build-up of the remaining two chiral centers with the help of the two already existing chiral centers (synthon A and B) by a highly diastereoselective halolactonisation. Final part, lactone aminolysis for integration of synthon C (*Herold et al, 2001; Herold and Stutz, 2001 and 2002; Herold et al, 2002[a] and 2002[b] and Stutz and Herold, 2002*). The synthon approach is simpler, flexible and less expensive manufacturing costs.

2.4.3 Physicochemical properties

ALS is a single diastereomer having four chiral centers, all S-configured, presented as a white to slightly yellowish crystalline powder. It is a hemifumarate salt of the

corresponding amine, with a molecular weight of 609.8 g/mol (551.8 g/mol without salt). It is molecular formula is $C_{30}H_{53}N_3O_6 \times 0.5\ C_4H_4O_4$ and has as its free base (pKa = 9.49). ALS is a hydrophilic molecule (log $P_{oct/water}$ = 2.45 at pH 7.4) with solubility in phosphate buffer, n-octanol and highly soluble in water (> 350 mg/ml at pH 7.4) (*Wood et al, 2003*).

Matsauka *et al.* have studied the conformational behavior of ALS in water, trifluoroethanol and DMF solutions using high-resolution nuclear magnetic resonance spectroscopy and molecular dynamics simulations. The results showed that ALS in aqueous solution adapts a U-shape conformation, whereas in DMF, the molecule is basically endowed with an extended conformation (*Matsauka et al, 2011*).

2.4.4 Mechanism of action

The renin-angiotensin-aldosterone system (RAAS) is a major target site for many antihypertensive agents, including angiotensin-converting enzyme inhibitors (ACE-I), angiotensin receptor blockers (ARBs) and aldosterone inhibitors (*Lam and Choy, 2007*). ALS binds with high specificity to the proteolytic active sites of renin enzyme to prevent cleaves of angiotensinogen to angiotensin I inactive form; the first and rate-limiting step of RAAS, which is converted to the active form angiotensin II by the ACE and non-ACE pathways figure (2.5). Angiotensin II has both direct and indirect effects on blood pressure. It is a powerful vasoconstrictor which directly causes arterial smooth muscle to contract and increase blood pressure. Furthermore, it stimulates the production of catecholamines and mineralcorticoid aldosterone from the adrenal cortex, which causes the tubules of the kidneys to increase reabsorption of sodium, with water following thereby increasing plasma volume and blood pressure. Moreover, angiotensin II regulates the level of renin in blood through a negative feedback mechanism (*Wood et al, 2003; Saseen and Carter, 2005 and Staessen et al, 2006*).

Unlike ACE-I and ARBs, which indirectly increase plasma renin activity, ALS directly inhibits renin to decrease plasma rennin activity, leading to reduced angiotensin I and angiotensin II levels (*Lam and Choy, 2007*). Increases in plasma rennin activity have been associated with four- to six-times higher mortality rates due to heart attacks and accelerated renal failure (*Alderman et al, 1991 and Alderman et al, 1997*).

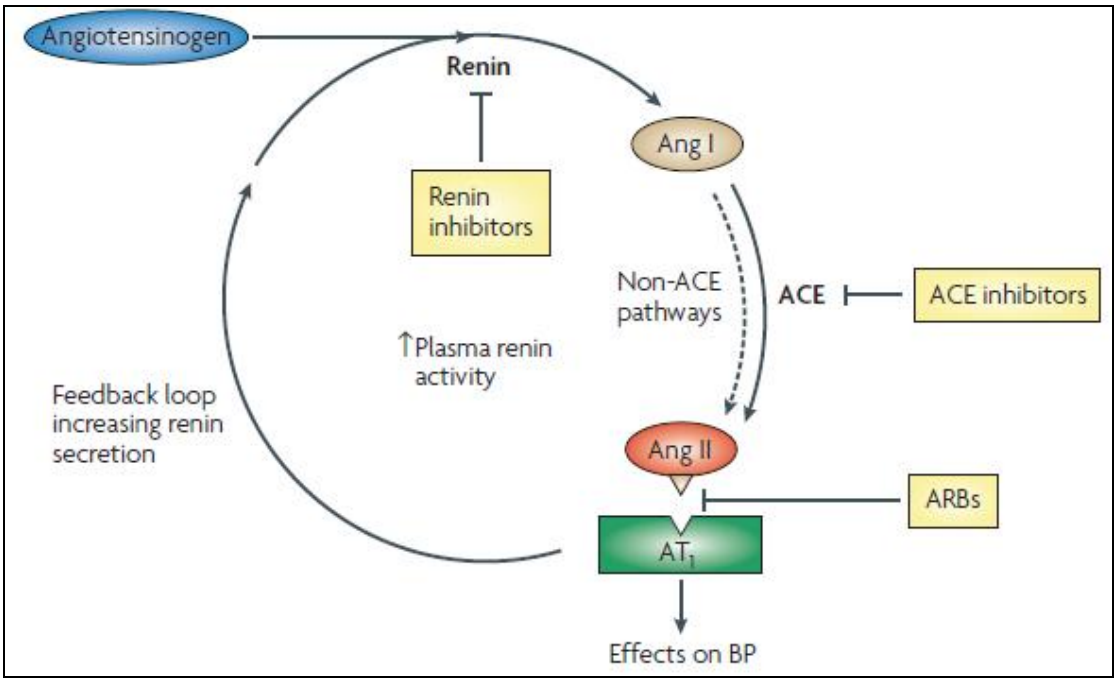

Figure 2.5: Schematic of the renin–angiotensin system.

2.4.5 Pharmacokinetics

ALS is orally active with a very low bioavailability (~2.5%) of the dose being absorbed. It reaches the peak plasma concentration within 1–6 hours after oral administration. ALS displays linear pharmacokinetics across a dose range of 75 to 600 mg (*Nussberger et al, 2002 and Novartis, 2007*). ALS has a volume of distribution <2 L/kg. It has a half-life of approximately 24 hours; thus steady-state blood levels can be expected in about 5–8 days (*Azizi et al, 2004; Wood et al, 2005 and Novartis, 2007*). ALS has modest water solubility with moderate protein binding (50%). However, it is detectable in the kidneys for up to 3 weeks after discontinuation of therapy; its plasma levels become undetectable at an earlier time (*Azizi et al, 2006*). ALS is metabolized by cytochrome P450 enzyme 3A4 (*Novartis, 2007*). More than 90% of ALS is eliminated unchanged in the feces, <2% is eliminated as oxidized metabolites, and <1% is eliminated in the urine (*Kobrin et al, 1993 and Nussberger et al, 2002*) and the estimated elimination half-life was 40 hours. Its pharmacokinetics is not affected by race, renal, or hepatic insufficiencies (*Novartis, 2007 and Vaidyanathan et al, 2007*). Diabetic patients have a higher drug exposure due to a slower drug clearance (205 vs. 234 L/h) and a longer elimination half-life (44 vs. 40 hours) (*Zhao et al, 2006*). However, the results of trial studies indicated that there was neither need for dose adjustments of ALS in patients with hepatic and renal diseases, nor for elderly and diabetic patients (*Jensen et al, 2008*).

2.4.6 Indication and dose

ALS is approved by FDA as monotherapy or combination therapy for hypertension. The recommended starting dose is 150 mg once daily, which may be increased to 300 mg daily to target blood pressure control (*Novartis, 2007 and Jensen et al, 2008*).

The safety and efficacy of ALS in various patient populations (elderly, diabetic, obese patients and patients with reduced renal and hepatic function) revealed that, there is no need for dosage adjustment (*Jensen et al, 2008*).

2.4.7 Adverse events

The most common adverse events in patients treated with ALS compared with those treated with placebo include diarrhea, cough, peripheral edema, fatigue, rash and influenza. The overall number of adverse events was lower in patients treated with ALS than in patients treated with placebo (37.7% versus 40.2%). Among these adverse events, diarrhea was the most common and was two-times more frequent in patients treated with ALS than patients treated with placebo (2.4% versus 1.2%). Cough, the second most common adverse event, was substantially less frequent in patients treated with ALS compared with patients treated with ACE-I (1.0% versus 3.8%), and peripheral edema was substantially less frequent in patients treated with ALS compared with those treated with amlodipine (0.9% versus 7.3%) (*Jensen et al, 2008*).

Transient elevations in serum potassium, above 5.5 mmol per L, have been observed with ALS. Mild increases in potassium are expected with agents that block the RAAS (including ACE-I and ARBs) by multiple mechanisms, including sudden changes in both glomerular filtration rate and aldosterone secretion (*Katzung, 2007*).

2.4.8 Contraindications and precautions

ALS is contraindicated in pregnancy as well as ACE-I and ARBs. Angioedema, hyperkalemia and hypotension may occur at any time during therapy, especially when used in combination with other antihypertensive agents. However, ALS does not require dose adjustment based on renal function, but caution should be exercised when used in patients with renal dysfunction (serum creatinine $\geq$ 1.7 mg/dL for women; $\geq$ 2.0 mg/dL for men and/or estimated glomerular filtration rate < 30 ml/min), a history of dialysis, nephrotic syndrome, or renovascular hypertension (*Novartis, 2007*).

2.4.9 Drug - drug interactions

ALS does not interact with the cytochrome P 450 system, so it would not have significant interactions with agents that are metabolized by this system. Moreover, it has no known

clinically relevant interactions with commonly used medicines for the treatment of hypertension or diabetes (*EMA, 2007 and Vaidyanathan, 2005*).

In clinical trials, there was no adjustment of furosemide dose when co-administered with ALS. However, ALS led to 30% decrease in furosemide area under the curve (AUC) and a 50% decrease in maximum plasma concentration ($C_{max)}$ in healthy subjects following single doses, when administered together (*Zhao et al, 2007*).

Co-administration of ALS with valsartan, metformin, amlodipine or cimetidine resulted in a 20–30% change in C_{max} or AUC of ALS. Atorvastatin administration with ALS led to steady-state ALS AUC and C_{max} increased by 50%. However, ALS had no significant effect on atorvastatin, valsartan, metformin or amlodipine pharmacokinetics (*Vaidyanathan et al, 2006*).

The European Medicines Agency has concluded that no dose adjustments for ALS or these co-administered medicinal products are necessary (*EMA, 2007*).

2.4.10 Assay

There is no official analytical method for analysis of ALS. Literature survey revealed that some analytical methods for determination of ALS in bulk and pharmaceutical dosage forms have been reported.

Pachauri *et al.* have developed HPLC method with photo diode array (PDA) detector for determination of ALS, ramipril, valsartan and hydrochlorothiazide in solid dosage forms. The quantitative determination of analyte(s) was performed on a normal phase C_{18} column. All analysis was carried out at a temperature of 40±2 °C under gradient conditions. The mobile phase consisted of a mixture of 0.2% v/v Triethylamine buffer (pH 3.0, adjusted with diluted ortho-phosphoric acid): Acetonitrile. The flow rate was 1.0 ml/min, the volume of injection was 20 µL, and all chromatograms were monitored in 200-400 nm range. The retention time of ALS was found to be 10.952 min and the correlation coefficient was 0.9993 for ALS calibration curve with accepted validation results (*Pachauri et al, 2010*).

Swamy *et al.* have developed three reverse-phase HPLC methods for determination of ALS alone, ALS in combination and ALS with other antihypertensive drugs in pharmaceutical dosage forms. The first method for quantification of ALS in bulk and tablet dosage form was performed with UV/Vis detector. The assay was performed using a C_{18} reversed-phase column eluted with phosphate buffer pH 3.0: Acetonitrile (60:40%, v/v) at a flow rate of 1.0 ml/min. The analysis was carried out at ambient temperature and detection was made at 293 nm. The retention time was 5.02 min and the linear

concentration range was 5-30 µg/ml with correlation coefficient of 0.9999 (*Swamy et al, 2011*). The second method was performed for simultaneous estimation of ALS and valsartan from their combination dosage form (Valturna®) using UV detector. Chromatographic separation was achieved isocratically on a Hiber Lichrospere® C_{18} column using a mobile phase of methanol, potassium dihydrogen phosphate buffer and acetonitrile (adjusted to pH 3.0 with 1% orthophosphoric acid) in the ratio of 50:30:20% v/v/v. The flow rate was 1.0 ml/min and the column was maintained at ambient temperature. The UV detection was carried out at 271 nm for ALS and valsartan, respectively. The retention time for ALS was 6.92 min and the method was found linear over the range of 10-50 µg/ml with correlation coefficient of 0.999 (*Swamy et al, 2012*[a]). The last method has been developed for the simultaneous estimation of ALS hemifumarate, amlodipine besylate and hydrochlorothiazide in their combination pharmaceutical dosage form (Amturnide®) with UV detector. Compounds were separated on Hiber Lichrosphere® C_{18} column under reversed phase partition conditions. The mobile phase was a 20:50:30% v/v/v mixture of acetonitrile: methanol: phosphate buffer (50Mm, pH 3±0.1, adjusted with orthophosphoric acid). The flow rate was 1.0 ml/min and chromatography was performed in ambient temperature maintained at 20±1 °C. All analytes were absorbed well at 239 nm. The retention time for ALS was found to be 6.85 min and linearity was obtained in the concentration range of 2-12 µg/ml with correlation coefficient of 0.9996 and suitable validation results (*Swamy et al, 2012*[b]).

Babu *et al.* have developed another reverse-phase HPLC method with dual mode wavelength detector to determine ALS hemifumarate in tablet dosage forms. The mobile phase was consisted of 0.03% trifluoroacetic acid (TFA) in water (solvent A) and 0.03% TFA in acetonitrile and water (95:5) (solvent B) and separation was achieved on a Waters Xbridge reverse phase C_{18} column with 10% B from 0 to 2.5 min, linearity increased to 95% B from 2.5 min to 8.50 min and was held until 10.50 min before returning to starting condition within remaining 4.5 min. The flow rate was 0.8 ml/min. Total run time was 15 min and the retention time of ALS was 9.46 min. ALS was monitored at the detection wavelength of 230/254 nm and the linear concentration range was 1-100 µg/ml with a correlation coefficient of 0.999 (*Babu et al, 2011*).

Aydogmus *et al.* have developed a spectrofluorimetric method for ALS determination in tablets and spiked human plasma based on derivatization reaction. This method was based on the reaction of the drug with dansyl chloride in the presence of bicarbonate solution of pH 10.5 to give a fluorescent derivative which was measured at 501 nm with excitation at

378 nm in dichloromethane as extracted solvent. The different experimental parameters affecting the development of the reaction product and its stability were studied and optimized. These factors include pH, type of buffer, amounts of reagent, temperature, reaction time and effect of extraction solvent. Such factors were changed individually while others were kept constant. The linear concentration range was 100-700 ng/ml with correlation coefficient of 0.9998 with suitable specificity, accuracy, precision, and robustness (*Aydogmus et al, 2011*).

Sangoi *et al.* have developed a stability-indicating micellar electro kinetic chromatography (MEKC) method with PDA for simultaneous determination of ALS and hydrochlorothiazide in pharmaceutical dosage forms. All experiments were carried out on a fused-silica capillary with 50 µm i.d and 40 cm of effective length. The optimized operating conditions were 47 mM Tris buffer and 47 mM sodium dodecyl sulfate as background electrolyte, pH 10.2, voltage 26 KV, capillary temperature 28 °C, hydrodynamic injection 5s at 50 mbar and the detection set at 217 nm. Linearity was found in the concentration range of 60-1200 µg/ml for ALS with correlation coefficient of 0.9999 (*Sangoi et al, 2011*).

Wrasse-Sangoi *et al.* have developed two analytical methods for determination of ALS in tablet dosage forms. The first method was UV spectrophotometric determination of ALS in pharmaceutical dosage form. A double-beam UV/Vis spectrophotometer, with a fixed slit width (2 nm) using 1.0 cm quartz cells was used for all absorbance measurements. The absorbance was measured at 279 nm. The method was linear in the range of 40-100 µg/ml with correlation coefficient of 0.9997 and suitable validation results (*Wrasse-Sangoi et al, 2010*). The second method was stability-indicating reverse-phase liquid chromatography with PDA detector. The method was carried out on a Waters Xbridge C_{18} column maintained at 25 °C. The mobile phase was consisted of acetonitrile: water (95:5%, v/v) / phosphoric acid (25 mM, pH 3.0) (40:60%, v/v) with flow rate of 1.0 ml/min and the PDA was set at 229 nm. The stability-indicating capability of the method was determined by subjecting a reference sample solution (200 µg/ml) to accelerated degradation by acidic (2 M hydrochloric acid, at ambient temperature for 5 h, then neutralized with base), basic (2 M NaOH, at ambient temperature for 5 h, then neutralized with acid), neutral (heated the drug which dissolved in water at 50°C for 96 h), oxidative (induced by storing the sample solution in 10% hydrogen peroxide, at ambient temperature for 30 h, protected from light) and photolytic (induced by exposing the sample in quartz cuvette to 200 watt h/square meter of near UV light at 25 cm of distance for 1 h) conditions to evaluate the interference

in the quantitation of ALS and was established by determining the peak purity of the samples. During the forced degradations, the neutral heated hydrolysis resulted in significant decrease of the area without any additional peak, indicating that the probable degradation products were not detected by UV. Under the acidic hydrolysis, ALS content exhibited a decrease of the area (7.94%) and one additional peak detected at 8.21 min. Just one additional peak was also detected in the oxidative condition at 5.05 min with significant decrease of ALS peak. Under the basic hydrolysis, nearly 69% of the ALS was degraded and three additional peaks were identified at 5.05, 6.15, and 7.87 min. For the photolytic condition, 59.76% of the ALS was degraded and five additional peaks were detected between 2.5 and 3.5 min, but these peaks did not interfere in the ALS peak purity. The retention time for ALS was 3.68 min and linearity was found in the concentration range of 10-300 µg/ml with correlation coefficient of 0.9999 and the method was showing adequate specificity, accuracy, precision and robustness (*Wrasse-Sangoi et al, 2011*).

2.5 Validation of an analytical method

Analytical methods are intended to establish the identity, purity, physical characteristics and potency of the drugs. Methods are developed to support drug testing against specifications during manufacturing and quality release operations, as well as during long-term stability studies. Methods may also support safety and characterization studies or evaluations of drug performance (*Swartz and Krull, 1997*).

Analytical method development and validation play a major role in the discovery, development, and manufacture of pharmaceuticals. Method validation is defined as the "process of demonstration that analytical procedures are suitable for their intended purpose" (*ICH, 2005*). The analytical procedure is 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 formula for the calculation, etc (*ICH, 2005*).

According to the ICH guidelines, the most common types of analytical procedures which need validation are: (i) identification tests, which intended to ensure the identity of an analyte in a sample; (ii) quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product (assay), which intended to measure the analyte present in a given sample. On the other hand, it represents a

quantitative measurement of the major component(s) in the drug product; (iii) quantitative tests for impurities' content and (iv) limits tests for the control of impurities.

Requirements for method validation are clear for new drug applications and many other worldwide marketing applications. These requirements are specified in documents from the ICH guidelines (*ICH, 2005*) and pharmacopeias (*USP, 2004*). It allows the analyst to understand the behavior of the method and to establish the performance limits of the method. Typical validation characteristics which should be considered are mentioned below according to ICH guidelines (*ICH, 2005*).

2.5.1 Linearity

The linearity of an analytical procedure is to obtain a linear response of results versus concentration (amount) of analyte (within a given range). For the establishment of linearity, a minimum of 5 concentrations is recommended.

2.5.2 Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

2.5.3 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. In other words, it's an assessment of the difference between the measured value and the true value.

2.5.4 Specificity

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

2.5.4.1 Identification

Suitable identification tests are to ensure the identity of an analyte presence and to discriminate between compounds of closely related structures which are likely to be present.

2.5.4.2 Assay and impurity test(s)

These tests are to ensure that all the analytical procedures performed allow an accurate statement of the content of an analyte in the presence of impurities and/or excipients.

2.5.5 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).

2.5.5.1 Repeatability

Its expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

2.5.5.2 Intermediate precision

Intermediate precision is a measure of precision within the same laboratory by different operators, using different instruments and making measurements on different days. It's also referred as inter-assay precision.

2.5.5.3 Reproducibility

Reproducibility expresses the precision between laboratories. In other words, it's the ability to give similar response for the same amount of analyte at different periods of time in different laboratories.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

2.5.6 LOD

The detection limit of an analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. Detection limit are based on visual evaluation, signal-to-noise, standard deviation of the response and the slope, standard deviation of the blank or based on the calibration curve.

2.5.7 LOQ

The quantitation limit of an 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. It's based on visual evaluation, signal-to-noise, standard deviation of the response and the slope, standard deviation of the blank or based on the calibration curve.

2.5.8 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its

reliability during normal usage. If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure.

Chapter 3

METHODOLOGY

3.1 Study design

This study is analytical experimental work. ALS will be under experimentation methods for spectrophotometric quantitative determination after derivatization reaction with NQS and NIN.

3.2 Ethical consideration

This study does not deal with human, so ethical consideration is not required.

Name of companies will not be published.

3.3 Limitation of the study

Lack of fund and the high cost of materials and equipments, as well as the limited resources such as books and journals were obstacles in the study. Israeli siege on Gaza Strip, also leads to delay or unavailability of chemicals and instruments.

3.4 Instruments and materials

3.4.1 Instruments

1. UV/Vis spectrophotometer

(SHIMADZU UV-1601 with UV-Pro software) and (PerkinElmer Lambda 25 with V5 ES software).

2. UV-lamp.

3. Water bath.

4. PH meter (SHIMADZU).

3.4.2 Materials and reagents

1. Standards: aliskiren (as a donation from NOVARTIS, Egypt), 1,2-naphthoquinone-4-sulfonic acid sodium salt (SEARLE Company, England), ninhydrin (Sigma-Aldrich Laborchemikalien GmbH, Germany).

2. Solvents: acetone, ethanol, methanol, isopropanol, acetonitrile, acetic acid, hydrochloric acid and distilled water. Solvents were delivered from FRUTAROM-LTD, Gaza, from different sources such as Zant company and Advance Tech.

3. Substances: ascorbic acid, sodium molybdate, zinc chloride, sodium hydroxide, citric acid, potassium dihydrogen phosphate, sodium hydrogen phosphate, sodium bicarbonate, ortho-phosphoric acid, magnesium stearate, titanium dioxide, talc, starch, glucose, lactose, povidone. All substances were proanalysis (P.A) grade.

4. Materials: Cuvette (Quartz), graduated pipette, volumetric flasks of different sizes (10, 50, 100, 250 and 500 ml) were purchased from MERK (Germany).

3.5 Data analysis

Data analysis was carried out by using statistical package of social science (SPSS) program version 16 (*SPSS, 2007*) as follows:

1. Data coding and entry.
2. Statistical examinations (One way ANOVA and *t*-test).
3. The significance of the results was determined according to the p-value.

3.6 Method I (NQS derivatization method)

3.6.1 Reagents

All employed chemicals were of analytical grade and high-purified water was used throughout the study.

3.6.1.1 NQS solution 0.5% (w/v)

250 mg of NQS was accurately weighed and transferred into 50 ml volumetric flask, dissolved in 10 ml distilled water and complete up the volume with distilled water. The solution was freshly prepared and protected from light during the use.

3.6.1.2 0.01 M NaOH solution

200 mg of NaOH was accurately weighed and transferred into a 500 ml volumetric flask, dissolved and completed the volume with distilled water.

3.6.2 Standard solution

ALS stock solution (1mg/ml) was prepared by dissolving 100 mg of ALS base powder in 100 ml distilled water. Working solutions were prepared by dilution the stock solution. The stock solution was freshly prepared during the use.

3.6.3 Selection of analytical wavelength for ALS-NQS chromogen

Two 10 ml volumetric flasks were taken and to each one, 1 ml 0.5% NQS solution and 1 ml 0.01 M NaOH were added and then 0.5 ml and 1 ml ALS solution (1mg/ml) were added to obtain 50 and 100 µg/ml as final concentrations, respectively. After the development of the orange color (after 10 min) at room temperature, the flasks were completed with distilled water. The absorption spectra and absorption maxima of ALS-NQS chromogen were determined against a blank was prepared from 1 ml 0.5% NQS solution and 1 ml 0.01 M NaOH without ALS.

3.6.4 Optimization of ALS-NQS reaction conditions

All of these measurements were made in triplicate, and the mean of each variable was calculated.

3.6.4.1 Effect of NQS concentration

The effect of varying NQS concentration was carried out using reagent concentration of 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 1%. 1 ml of each reagent concentration was mixed with 1 ml 0.01 M NaOH and 0.5 ml ALS solution (1mg/ml) in 10 ml volumetric flasks at room temperature. The volume was completed with distilled water after 10 min. The absorbances of the products formed were measured at 500 nm against a blank was prepared for each tested concentration percent of NQS with 1 ml 0.01 M NaOH without ALS.

3.6.4.2 Effect of volume of 0.5% NQS solution

The effect of 0.5% (w/v) NQS volume was carried out using different volumes of 0.5, 1, 1.5, and 2 ml. Each of these volumes was mixed with 1 ml 0.01 M NaOH and 0.5 ml ALS solution (1mg/ml) at room temperature in 10 ml volumetric flasks. The volume was completed with distilled water after 10 min. The absorbance was measured at 500 nm against a blank was prepared for each tested volume with 1 ml 0.01 M NaOH without ALS.

3.6.4.3 Effect of alkalinity

Different inorganic bases were tested; NaOH, sodium hydrogen phosphate, and sodium bicarbonate. All were prepared as aqueous solutions of concentration 0.01, 0.02, 0.03, 0.04, 0.1, and 1 M. 1 ml of each solution was mixed with 1 ml 0.5% NQS solution and 0.5 ml ALS solution (1mg/ml) in 10 ml volumetric flasks at room temperature. The volume was completed with distilled water after 10 min. The absorbance was measured at 500 nm against blank was prepared for each tested base at different concentration with 1 ml 0.5% NQS without ALS. Furthermore, we tested the importance of alkalinity in the reaction by carrying out the reaction without any inorganic bases; just added 1 ml 0.5% NQS solution to 0.5 ml ALS solution (1mg/ml) in 10 ml volumetric flask at room temperature. After 10 min the volume was completed with distilled water and the absorbance was measured at 500 nm against a blank solution was prepared from 1 ml 0.5 NQS diluted up to 10 ml.

3.6.4.4 Effect of volume of 0.01 M NaOH solution

The effect of 0.01 M NaOH volume was carried out using different volumes of 0.5, 1, 1.5, and 2 ml. Each of these volumes was mixed with 1 ml 0.5% NQS solution and 0.5 ml ALS solution (1mg/ml) at room temperature in 10 ml volumetric flasks. The volume was

completed with distilled water after 10 min. The absorbance was measured at 500 nm against a blank was prepared for each tested volume with 1 ml 0.5% NQS without ALS.

3.6.4.5 Effect of pH

The influence of pH on the development of ALS-NQS chromogen was investigated by carrying out the reaction in 0.1 M phosphate buffer of different pH values (*Smith, 2007*).

1 ml of 0.1 M phosphate buffer systems of (3, 4.4, 5.1, 6.32, 7.1, 8.3, 9.63, 10.7, 11.4, 12.63 and 13.7 pH values) was mixed with 1 ml 0.5% NQS solution and 0.5 ml ALS solution (1mg/ml) at room temperature in 10 ml volumetric flasks. The volume was completed with distilled water after 10 min. The absorbance was measured at 500 nm against a blank, which was prepared for each tested pH of buffer system with 1 ml 0.5% NQS without ALS.

3.6.4.6 Effect of diluting solvent

In order to select the most appropriate solvent for diluting the reaction solution, different solvents were tested; methanol, ethanol, isopropanol, acetone, acetonitrile, methyl acetate, and water. Each of these solvents was used for the final dilution of the reaction after 10 min from adding 1 ml 0.5% NQS solution to 1 ml 0.01 M NaOH and 0.5 ml ALS solution (1mg/ml) in 10 ml volumetric flasks at room temperature. The absorbance was measured at 500 nm against a blank was prepared for each diluting solvent with 1 ml 0.5% NQS solution and 1 ml 0.01 M NaOH without ALS.

3.6.4.7 Effect of temperature and time

The effect of temperature and time on the reaction was studied by carrying out the reaction at different temperatures (25-60 °C) for (5-15 min). 1 ml 0.5% NQS solution, 1 ml 0.01 M NaOH and 0.5 ml ALS solution (1mg/ml) were mixed in 10 ml volumetric flask. The mixture was allowed to stand at room temperature (25 °C) and heated at different temperature; 40, 50 and 60 ± 3 °C. The period for each tested temperature was 5, 10 and 15 min. After cooling to room temperature the mixture was diluted with distilled water and the absorption at 500 nm was measured against a blank was prepared for each tested temperature and time with 1 ml 0.5% NQS solution and 1 ml 0.01 M NaOH without ALS.

3.6.4.8 Order of addition effect

The effect of reagent addition was carried out in different orders as described in the table 3.1.

Table 3.1: Order of addition of NQS method.

First	Second	Third
NQS	NaOH	ALS
NQS	ALS	NaOH
NaOH	NQS	ALS
NaOH	ALS	NQS
ALS	NQS	NaOH
ALS	NaOH	NQS

1 ml 0.5% NQS solution, 1 ml 0.01 M NaOH and 0.5 ml ALS solution (1mg/ml) in different orders were mixed in 10 ml volumetric flasks. The mixture was allowed to stand for 10 min at room temperature and then was diluted with distilled water. The absorbance was measured at 500 nm against a blank was made in the same order of the test with 1 ml 0.5% NQS solution and 1 ml 0.01 M NaOH without ALS.

3.6.4.9 Stability of ALS-NQS chromogen

The effect of time on the stability of ALS-NQS chromogen was studied by following the absorption intensity of the reaction solution (after dilution) at different time intervals up to 4 hours.

3.6.4.10 Determination of stoichiometric ratio

3.6.4.10.1 Limiting logarithmic method

The limiting logarithmic method (*Rose, 1964*) was employed. Two sets of experiments were carried out employing the general procedures described above. The first set of experiments was carried using varying concentrations of NQS ($1.9 \times 10^{-3} – 19.2 \times 10^{-3}$ M) at a fixed ALS concentration (1.8×10^{-4} M). The second set of experiments was carried using varying concentrations of ALS ($0.4 \times 10^{-4} – 5 \times 10^{-4}$ M) at a fixed concentration of NQS (1.9×10^{-2} M). The logarithms of the obtained absorbances for the reaction of ALS with NQS were plotted as a function of the logarithms of the concentrations of NQS and ALS in the first and second sets of experiments. The slopes of the fitting lines in both sets of experiments were calculated.

3.6.4.10.2 Job's method

The Job's method of continuous variation (*Job, 1964*) was employed. Equimolar (5×10^{-3}) aqueous solutions of NQS and ALS were prepared. Series of 1 ml portions of the solutions of NQS and ALS were made up comprising different complementary proportions (0:1,

0.1:0.9, 0.2:0.8, 0.3:0.7, 0.4:0.6, 0.5:0.5, 0.6:0.4, 0.7:0.3, 0.8:0.2, 0.9:0.1 and 1:0) in 10 ml volumetric flasks, respectively. The solutions were carried out employing the general procedures described above. The mole fraction of NQS were calculated and plotted versus absorbances obtained. The mole ratio of NQS and ALS was determined.

3.6.5 Validation of NQS method

3.6.5.1 Linearity

0.2, 0.5, 1, 1.5, 2, 2.5 and 3 ml of ALS solution (1mg/ml) were transferred into a series of 10 ml volumetric flasks to have final concentration ranged from 20-300 µg/ml. To each solution, 1 ml 0.5% NQS and 1 ml 0.01 M NaOH were added and mixed gently. The mixtures were allowed to stand at room temperature for 10 min. The mixtures were diluted with distilled water and the absorbances at 500 nm were measured against blank solution was prepared from 1 ml 0.5% NQS and 1 ml 0.01 M NaOH without ALS.

The concentrations of ALS 20, 50, 100, 150, 200, 250 and 300 µg/ml were plotted versus absorption. The regression line and correlation coefficient were evaluated, as well as the validity of regression line was verified by statistical analysis.

3.6.5.2 Range

The specified range is normally derived from linearity studies and it is the interval between the upper and lower concentration of the ALS in the sample.

3.6.5.3 Accuracy

Accuracy was determined by adding known quantities of the ALS standard (50%, 100% and 150%, w/v) to a preanalyzed drug product of three different concentrations (50, 80, and 100 µg/ml) and determined as percent recovery by the following equation;

% Recovery = [(Ct-Cs) / Ca] x 100

Ct: total drug measured after standard addition.

Cs: drug concentration in the formulation sample.

Ca: drug concentration added to formulation.

3.6.5.4 Specificity

Specificity according to ICH guidelines (*ICH, 2005*), consists of identification test and the assay of the analyte.

3.6.5.4.1 Identification

The identification test was confirmed by obtaining positive results from samples containing the ALS, coupled with negative results from samples which do not contain ALS. This was achieved by comparing the absorption at the same wavelength of ALS-NQS chromogen against blank versus blank against water.

3.6.5.4.2 Assay

The assay of the method was demonstrated by adding appropriate levels of excipients to the drug and calculating the percent of drug recovery. Samples were prepared by mixing 50 and 100 mg of standard ALS powder with various amounts of common excipients such as glucose 50 mg, lactose 50 mg, starch 50 mg, talk 50 mg, titanium dioxide 50 mg, povidone 10 mg and magnesium stearate 10 mg in several 100 ml volumetric flasks. After mixing and filtration of each solution, 1 ml of each solution was mixed with 1 ml 0.5% NQS solution and 1 ml 0.01 M NaOH in 10 ml volumetric flask at room temperature. Then procedures were continued as described previously.

3.6.5.5 Precision

3.6.5.5.1 Repeatability

Repeatability was assessed using three different concentrations; 50, 150, 250 µg/ml of the drug. Five replicates were measured for each concentration. The relative standard deviation (RSD) was calculated for each concentration.

3.6.5.5.2 Intermediate Precision

The effect of random events on the precision of the analytical procedure was studied in different days by repeating the procedure for different concentrations; 50, 150, 250 µg/ml of the drug over six consecutive days. The RSD was calculated for each concentration.

3.6.5.6 LOD

The detection limit was expressed using the following formula *(ICH, 2005)*;

$LOD = 3.3\ \sigma / S$

σ: residual standard deviation of the regression line.

S: slope of the regression line.

3.6.5.7 LOQ

It is calculated from the following formula *(ICH, 2005)*;

$LOQ = 10\ \sigma / S$

σ: residual standard deviation of the regression line.

S: slope of the regression line.

3.6.5.8 Robustness

Robustness was examined by evaluating the influence of small variation in the method variables on its analytical performance. The variables tested were the concentration (w/v %) and volume of NQS, the molarity and volume of NaOH solution, temperature and time of the reaction. One parameter was changed while the others were kept unchanged and recovery percentage was calculated each time.

3.6.5.9 Ruggedness (inter-laboratory precision)

Ruggedness was assessed by means of different laboratories. It was assessed by replicating the procedure for three concentrations; 50, 150, 250 µg/ml of the drug three times in two different laboratories with different spectrophotometer (SHIMADZU UV-1601, Japan and PerkinElmer Lambda 25, England). The RSD was calculated for each concentration.

3.6.6 Analysis of commercial pharmaceutical product

The pharmaceutical dosage form was subjected to the analysis of their ALS content by NQS method and reference method (*Wrasse-Sangoi, 2010*). Ten tablets were weighed, crushed and their contents mixed thoroughly. An accurately weighed portion of powder equivalent to the labeled strength (100 mg) ALS was taken into a 100 ml volumetric flask containing about 75 ml of distilled water. This was shaken thoroughly for about 5–10 min, filtered with Whatman filter paper to remove insoluble matter and diluted with distilled water to prepare 1000 µg/ml solution. To the sample solution (0.5 ml), 1 ml 0.5 % NQS and 1 ml 0.01 M NaOH were added. The mixture was gently shaken and allowed to stand for 10 min at room temperature. The contents were diluted to 10 ml with distilled water. The absorbances were measured at 500 nm against blank solution. The result was compared with reference method result by statistical analysis (*t*-test).

3.7 Method II (NIN derivatization method)

3.7.1 Reagents

3.7.1.1 NIN solution 1.2% (w/v)

600 mg of NIN was accurately weighed and transferred into 50 ml volumetric flask, dissolved in distilled water with sonication and completed the volume with distilled water.

3.7.1.2 Ascorbic acid solution 0.1% (w/v)

50 mg of ascorbic acid was accurately weighed and transferred into 50 ml volumetric flask, dissolved and completed with distilled water.

3.7.1.3 0.2 M Phosphate buffer system pH 6.0

50 ml 0.2 M phosphate buffer prepared by dissolving 1.3609 gm potassium dihydrogen phosphate with distilled water in 50 ml volumetric flask and completed with distilled water. In 200 ml volumetric flask, add the previous solution mixed with 4 ml 1 M NaOH and completed the volume with distilled water to 190 ml then adjust the pH to 6.0 with 1 M NaOH solution with pH meter and completed the volume with distilled water.

3.7.1.4 1.2% NIN and 0.1% ascorbic acid in 0.2 M phosphate buffer pH 6.0 (N/A/PH)

600 mg of NIN powder with 50 mg of ascorbic acid were accurately weighed and dissolved in 50 ml 0.2 M phosphate buffer pH 6.0 as one solution.

3.7.2 Standard solution

ALS stock solution (2mg/ml) was prepared by dissolving 200 mg ALS base powder in distilled water in 100 ml volumetric flask and completed the volume with distilled water. Working solutions of the drug were prepared by dilution of the stock solution.

3.7.3 Selection of analytical wavelength for ALS-NIN chromogen

Two volumetric flasks 10 ml were taken and to each one, 1 ml N/A/PH solution and 1 ml of ALS solution (0.5mg/ml and 1mg/ml) were added to obtain 50 and 100 µg/ml as final concentrations, respectively. After incubation in water bath (90±3 °C) for 20 min, the contents were cooled with tap water until reacted room temperature and completed with distilled water. The absorption spectra and absorption maxima of ALS-NIN chromogen were determined against a blank was prepared from 1 ml N/A/PH solution without ALS.

3.7.4 Optimization of ALS-NIN reaction conditions

All of these measurements were made in triplicate, and the mean of each variable was calculated.

3.7.4.1 Effect of NIN concentration

The effect of varying NIN concentration was carried out using reagent concentration of 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.5, and 2%. 1 ml of each reagent concentration was mixed with 1 ml 0.2 M phosphate buffer pH 6.0 and 1 ml ALS solution (1mg/ml) in 10 ml volumetric flasks. The mixtures were incubated at 90±3 °C water bath for 20 min, then cooled with tap water to the room temperature and diluted with distilled water. The absorbance was measured at 569 nm against a blank was prepared for each tested concentration of NIN with 1 ml 0.2 M phosphate buffer pH 6.0 without ALS.

3.7.4.2 Effect of volume of 1.2% NIN solution

The effect of 1.2% (w/v) NIN volume was carried out using different volumes of 0.5, 1, 1.5, and 2 ml. Each of these volumes was mixed with 1 ml 0.2 M phosphate buffer pH 6.0 and 1 ml ALS solution (1mg/ml) for 20 min in 90±3 °C water bath in 10 ml volumetric flasks. The mixture was cooled to room temperature with tap water and completed with distilled water. The absorbance was measured at 569 nm against a blank was prepared for each tested volume with 1 ml 0.2 M phosphate buffer pH 6.0 without ALS.

3.7.4.3 Effect of buffer system type

Phosphate and citrate buffer systems were tested. Both were prepared in 0.2 M and were adjusted to pH 6.0 by 1 M NaOH. 1 ml of each buffer type was mixed with 0.5 ml 1.2% NIN solution and 1 ml ALS solution (1mg/ml) for 20 min in 10 ml volumetric flasks in 90±3 °C water bath. The mixture was cooled to the room temperature and completed with distilled water. The absorbance was measured at 569 nm against a blank was prepared for each tested buffer system with o.5 ml 1.2% NIN solution without ALS.

3.7.4.4 Effect of pH

The influence of pH on the development of ALS-NIN chromogen was investigated by carrying out the reaction in 0.2 M citrate buffer system (4.5, 5, 5.5, 6, and 6.5) and in 0.2 M phosphate buffer system (6, 6.5, and 7) pH values (*Smith, 2007*). 1 ml of each of them was mixed with 0.5 ml 1.2% NIN solution and 1 ml ALS solution (1mg/ml) for 20 min in 90±3 °C water bath in 10 ml volumetric flasks. The mixture was cooled to the room temperature and completed with distilled water. The absorbance was measured at 569 nm against a blank was prepared for each tested pH of each buffer system with 0.5 ml 1.2% NIN solution without ALS.

3.7.4.5 Effect of volume of 0.2 M phosphate buffer pH 6.0

The effect of 0.2 M phosphate buffer pH 6.0 volume was carried out using different volumes of 0.5, 1, 1.5, and 2 ml. Each of these volumes was mixed with 0.5 ml 1.2% NIN solution and 1 ml ALS solution (1mg/ml) for 20 min in 90±3 °C water bath in 10 ml volumetric flasks. The mixture was cooled to the room temperature with tap water and completed with distilled water. The absorbance was measured at 569 nm against a blank was prepared for each tested volume with 0.5 ml 1.2% NIN solution without ALS.

3.7.4.6 Effect of type and concentration of reducing agent

Different reducing agents were tested; ascorbic acid, sodium molybdate and zinc chloride. All were prepared as aqueous solutions of concentration 0.1, 0.2, and 1% (w/v). 1 ml of each solution was mixed with 0.5 ml 1.2% NIN solution, 1 ml 0.2 M phosphate buffer system pH 6.0 and 1 ml ALS solution (1mg/ml) for 20 min in 90±3 °C water bath in 10 ml volumetric flasks. The mixture was cooled to the room temperature with tap water and completed with distilled water. The absorbance was measured at 569 nm against a blank was prepared for each tested reducing agent at different concentration mixed with 0.5 ml 1.2% NIN solution and 1 ml 0.2 M phosphate buffer system pH 6.0 without ALS.

3.7.4.7 Effect of diluting solvent

In order to select the most appropriate solvent for diluting the reaction solution, different solvents were tested; methanol, ethanol, isopropanol, acetonitrile, ethyl acetate, and water. Each of these solvents was used for the final dilution of the reaction after 20 min from adding 0.5 ml 1.2% NIN solution to 1 ml 0.2 M phosphate buffer system pH 6.0 and 1 ml ALS solution (1mg/ml) in 10 ml volumetric flasks in 90±3 °C water bath. The mixture was cooled to the room temperature. The absorbance was measured at 569 nm against a blank was prepared for each diluting solvent with 0.5 ml 1.2% NIN solution and 1 ml 0.2 M phosphate buffer system pH 6.0 without ALS.

3.7.4.8 Effect of temperature and time

The effect of temperature and time on the reaction was studied by carrying out the reaction at different temperatures (80-100 °C) for (5-25 min). 0.5 ml 1.2% NIN solution, 1 ml 0.2 M phosphate buffer system pH 6.0 and 1 ml ALS solution (1mg/ml) were mixed in 10 ml volumetric flask. The mixture was heated at different temperatures; 80, 90 and 100 ± 3 °C. The heating period for each tested temperature was 5, 10, 15, 20 and 25 min. After cooling to room temperature the mixture was diluted with distilled water. The absorption was measured at 569 nm against a blank was prepared for each tested temperature and time with 0.5 ml 1.2% NIN solution and 1 ml 0.2 M phosphate buffer system pH 6.0 without ALS. Moreover, the importance of heating in water bath was investigated by carrying out the reaction without heating (in room temperature) for several hours.

3.7.4.9 Order of addition effect

The effect of reagent addition was carried out by testing several combination solutions.

1- 1 ml 1.2% NIN dissolved in 0.2 M phosphate buffer system pH 6.0.

2- 1 ml of first solution mixed with 1 ml 0.1% ascorbic acid dissolved in distilled water.

3- 1 ml 1.2% NIN with 0.1% ascorbic acid dissolved in 0.2 M phosphate buffer system pH 6.0 (N/A/PH).

4- 1 ml 1.2% NIN with 0.1% ascorbic acid dissolved in distilled water mixed with 0.5 ml 0.2 M phosphate buffer system pH 6.0.

5- 1 ml 1.2% NIN with 0.03% ascorbic acid dissolved in 0.2 M phosphate buffer system pH 6.0.

The five solutions were tested by determining the beer's law limits and calculating the correlation coefficients of obtained calibration curves. Moreover, the effect of N/A/PH solution volume was carried out using different volumes of 0.5, 1, 1.5 and 2 ml. Each of these volumes was mixed with 1 ml ALS solution (1mg/ml) for 20 min in 90±3 °C water

bath in 10 ml volumetric flasks. The mixture was cooled to the room temperature with tap water and completed with distilled water. The absorbance was measured at 569 nm against a blank was prepared for each tested volume without ALS.

3.7.4.10 Stability of ALS-NIN chromogen

The effect of time on the stability of ALS-NIN chromogen was studied by following the absorption intensity of the reaction solution (after dilution) at different time intervals up to 4 hours.

3.7.4.11 Determination of stoichiometric ratio

3.7.4.11.1 Limiting logarithmic method

The limiting logarithmic method (*Rose, 1964*) was employed. Two sets of experiments were carried out employing the general procedures described above. The first set of experiments was carried using varying concentrations of NIN ($3.9 \times 10^{-3} – 11 \times 10^{-3}$ M) at a fixed ALS concentration (1.8×10^{-4} M). The second set of experiments was carried using varying concentrations of ALS ($0.9 \times 10^{-4} – 3.6 \times 10^{-4}$ M) at a fixed concentration of NIN (6.7×10^{-3} M). The logarithms of the obtained absorbances for the reaction of ALS with NIN were plotted as a function of the logarithms of the concentrations of NIN and ALS in the first and second sets of experiments. The slopes of the fitting lines in both sets of experiments were calculated.

3.7.4.11.2 Job's method

The Job's method of continuous variation (*Job, 1964*) was employed. Equimolar (5×10^{-3}) aqueous solutions of NIN and ALS were prepared. Series of 1 ml portions of the solutions of NIN and ALS were made up comprising different complementary proportions (0:1, 0.1:0.9, 0.2:0.8, 0.3:0.7, 0.4:0.6, 0.5:0.5, 0.6:0.4, 0.7:0.3, 0.8:0.2, 0.9:0.1 and 1:0) in 10 ml volumetric flasks, respectively. The solutions were carried out employing the general procedures described above. The mole fraction of NIN were calculated and plotted versus absorbances obtained. The mole ratio of NIN and ALS was determined.

3.7.5 Validation of NIN method

3.7.5.1 Linearity

1 ml of ALS solutions (0.1, 0.2, 0.5, 0.8, 1.1, 1.4 and 1.7 mg/ml) were transferred into a series of 10 ml volumetric flasks to have final concentration ranged from 10-170 µg/ml. To each solution 1 ml N/A/PH solution was added. The mixtures were heated in water bath 90±3 °C for 20 min. The mixtures were cooled to room temperature with tap water and completed with distilled water. The absorbances at 569 nm were measured against a blank was prepared from 1 ml N/A/PH solution without ALS. The concentrations of ALS 10, 20,

50, 80, 110, 140 and 170 µg/ml were plotted versus absorption. The regression line and correlation coefficient were calculated. The validity of regression line was also verified by statistical analysis.

3.7.5.2 Range

The specified range is normally derived from linearity studies and it is the interval between the upper and lower concentration of the ALS in the sample.

3.7.5.3 Accuracy

Accuracy was determined by adding known quantities of the ALS standard (50%, 100% and 150%, w/v) to a preanalyzed drug product of three different concentrations (20, 40, and 60 µg/ml) and determined as percent recovery.

3.7.5.4 Specificity

3.7.5.4.1 Identification

Identification test was achieved by comparing the absorption at the same wavelength of ALS-NIN chromogen against blank versus blank against water.

3.7.5.4.2 Assay

The assay of the method was demonstrated by adding appropriate levels of excipients to the drug and calculating the percent of drug recovery. Samples were prepared by mixing 50 and 100 mg of standard ALS powder with various amounts of common excipients such as glucose 50 mg, lactose 50 mg, starch 50 mg, talk 50 mg, titanium dioxide 50 mg, povidone 10 mg and magnesium stearate 10 mg in several 100 ml volumetric flasks. After mixing and filtration of each solution, 1 ml of each solution was mixed with 1 ml N/A/PH solution for 20 min in 90±3 °C water bath in 10 ml volumetric flask. Then procedures were continued as described previously.

3.7.5.5 Precision

3.7.5.5.1 Repeatability

Repeatability was assessed using three different concentrations; 20, 80, 140 µg/ml of the drug. Five replicates were measured for each concentration. The RSD was calculated for each concentration.

3.7.5.5.2 Intermediate Precision

The effect of random events on the precision of the analytical procedure was studied in different days by repeating the procedure for three concentrations; 20, 80, 140 µg/ml of the drug over six consecutive days. The RSD was calculated for each concentration.

3.7.5.6 LOD

The detection limit was expressed using the following formula;

LOD = 3.3 σ / S

3.7.5.7 LOQ

It is calculated from the following formula;

LOQ = 10 σ / S

3.7.5.8 Robustness

Robustness was examined by evaluating the influence of small variation in the method variables on its analytical performance. The variables tested were the concentration (%, w/v) of N/A/PH solution, temperature and time of the reaction. One parameter was changed while the others were kept unchanged and recovery percentage was calculated.

3.7.5.9 Ruggedness (inter-laboratory precision)

Ruggedness was assessed by means of different laboratories. It was assessed by replicating the procedure for three concentrations; 20, 80, 140 µg/ml of the drug three times in two different laboratories with different spectrophotometer (SHIMADZU UV-1601, Japan and PerkinElmer Lambda 25, England). RSD was calculated for each concentration.

3.7.6 Analysis of commercial pharmaceutical product

The pharmaceutical dosage form was subjected to the analysis of their ALS content by NIN method and reference method (*Wrasse-Sangoi, 2010*). Ten tablets were weighed, crushed and their contents mixed thoroughly. An accurately weighed portion of powder equivalent to the labeled strength (100mg) of ALS was taken into a 100 ml volumetric flask containing about 75 ml of distilled water. This was shaken thoroughly for about 5–10 min, filtered with Whatman filter paper to remove insoluble matter and diluted with distilled water to prepare 1000 µg/ml solution. To the sample solution (0.5 ml), 1 ml N/A/PH solution was added. The mixture was heated in water bath 90±3 °C for 20 min. The mixtures were cooled with tap water to the room temperature and diluted to 10 ml with distilled water. The absorbances were measured at 569 nm against a blank was prepared from 1 ml N/A/PH without ALS. The result was compared with reference method result by statistical analysis (*t*-test).

3.8 Comparison between NQS, NIN and reference method

NQS, NIN and reference methods were compared in according to beer's law limits, maximum wavelength, correlation coefficients, molar absorptivity, LOD and LOQ. Reliability relationship between methods were tested by one way ANOVA test.

Chapter 4

RESULTS

4.1 Method I (NQS derivatization method)

4.1.1 Selection of analytical wavelength for ALS-NQS chromogen

The absorption spectra and maximum absorption point of the ALS-NQS chromogens of 50 and 100 µg/ml for ALS as final concentrations (figure 4.1) were determined against blank solution on the UV/Vis spectrophotometer. The maximum absorption point was 500 nm.

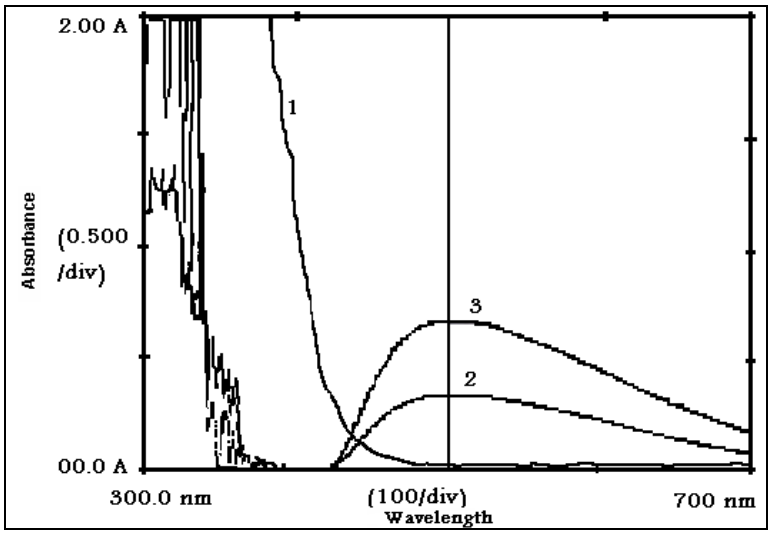

Figure 4.1: Selection of analytical wavelength of ALS-NQS chromogen, {1: blank spectrum against water; 2 and 3: chromogen against blank at ALS: 50 and 100 µg/ml}.

4.1.2 Optimization of NQS reaction conditions

4.1.2.1 Effect of NQS concentration

Table 4.1 and figure 4.2 show the effect of NQS concentration% on the reaction. The maximum absorption was obtained with 0.5% NQS solution.

Table 4.1: Effect of NQS concentration on NQS method.

Sample	NQS concentration% (w/v, 1ml)	Absorbance[a]
1	0.05	0.135
2	0.1	0.195
3	0.2	0.290
4	0.3	0.303
5	0.4	0.309
6	0.5	0.360
7	0.6	0.336
8	1	0.318

[a]: values were mean of three determinations; ALS 50µg/ml, 0.01M NaOH (1ml).

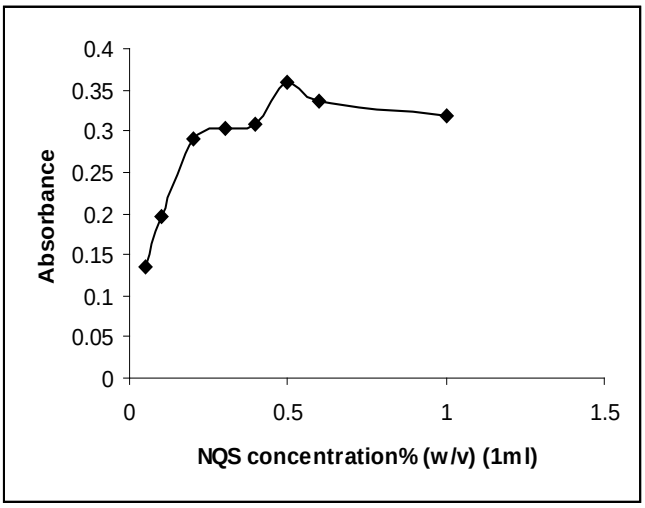

Figure 4.2: Effect of NQS concentration on NQS method; {ALS 50µg/ml, 0.01M NaOH (1ml)}.

4.1.2.2 Effect of 0.5% NQS solution volume

Table 4.2 show the effect of volume of 0.5% NQS solution on the reaction. The best volume was 1 ml.

Table 4.2: Effect of 0.5% NQS volume on NQS method.

Sample	0.5% NQS volume (ml)	Absorbance[a]
1	0.5	0.321
2	1	0.360
3	1.5	0.335
4	2	0.320

[a]: values were mean of three determinations; ALS 50µg/ml, 0.01M NaOH (1ml).

4.1.2.3 Effect of alkalinity

Table 4.3 show the effect of different inorganic bases on the reaction of ALS and NQS in different molarity. The best results obtained in case of 0.01 M NaOH.

Table 4.3: Effect of alkalinity on NQS method.

Sample	Type of inorganic base	Molar concentration (M)	Absorbance[a]
1	NaOH	0.01	0.360
2		0.02	0.343
3		0.03	0.332
4		0.04	0.313
5		0.1	0.235
6		1	0.150
1	$NaHCO_3$	0.01	0.231
2		0.02	0.248
3		0.03	0.294
4		0.04	0.326
5		0.1	0.332
6		1	0.317
1	$NaHPO_4$	0.01	0.300
2		0.02	0.310
3		0.03	0.319
4		0.04	0.335
5		0.1	0.326
6		1	0.335
The reaction did not proceed without alkaline media			

[a]: values were mean of three determinations; ALS 50µg/ml, 0.5% NQS (1ml).

4.1.2.4 Effect of 0.01 M NaOH solution volume

Table 4.4 show the effect of volume of 0.01 M NaOH on the reaction. The best volume was 1 ml.

Table 4.4: Effect of 0.01 M NaOH volume on NQS method.

Sample	0.01 M NaOH volume (ml)	Absorbance[a]
1	0.5	0.302
2	1	0.360
3	1.5	0.350
4	2	0.334

[a]: values were mean of three determinations; ALS 50µg/ml, 0.5% NQS (1ml).

4.1.2.5 Effect of pH

Table 4.5 and figure 4.3 show the effect of 0.1 M phosphate buffer system of varying pH values on the reaction without using inorganic bases. Maximum absorption was obtained in pH range 10-11.5.

Table 4.5: Effect of pH on NQS method.

Sample	pH of 0.1M phosphate buffer solution (1ml)	Absorbance[a]
1	3	0.000
2	4.4	0.004
3	5.1	0.006
4	6.32	0.072
5	7.1	0.178
6	8.3	0.311
7	9.63	0.334
8	10.7	0.350
9	11.4	0.354
10	12.63	0.312
11	13.7	0.3

[a]: values were mean of three determinations; ALS 50µg/ml, 0.5% NQS (1ml).

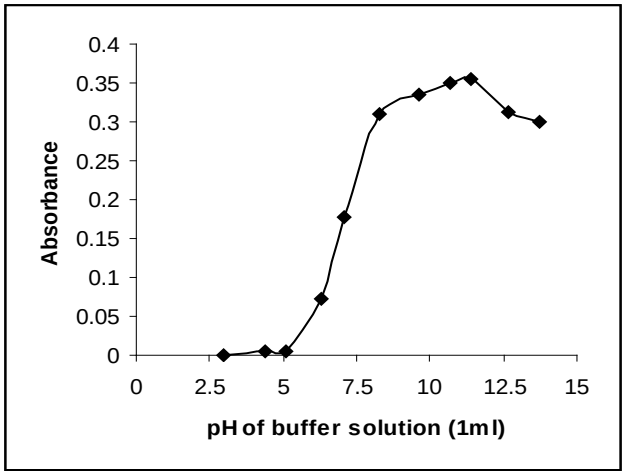

Figure 4.3: Effect of pH on NQS method; {ALS 50µg/ml, 0.5% NQS (1ml), H_2O diluting solvent, 10 min reaction time at room temperature}.

4.1.2.6 Effect of diluting solvent

Table 4.6 and figure 4.4 show the effect of using different solvents in the final dilution of the reaction.

High absorbances were obtained in case of acetone and methyl acetate, but without distinguish peaks. Water was used as diluting solvent.

Table 4.6: Effect of diluting solvent on NQS method.

Sample	Diluting solvent	Absorbance[a]
1	Methanol	0.319
2	Ethanol	0.313
3	Isopropanol	0.303
4	Acetonitrile	0.339
5	Acetone	1.395*
6	Methyl acetate	0.558*
7	Water	0.360

[a]: values were mean of three determinations, *: high readings without distinguish peaks; ALS 50µg/ml, 0.5% NQS (1ml), 0.01M NaOH (1ml).

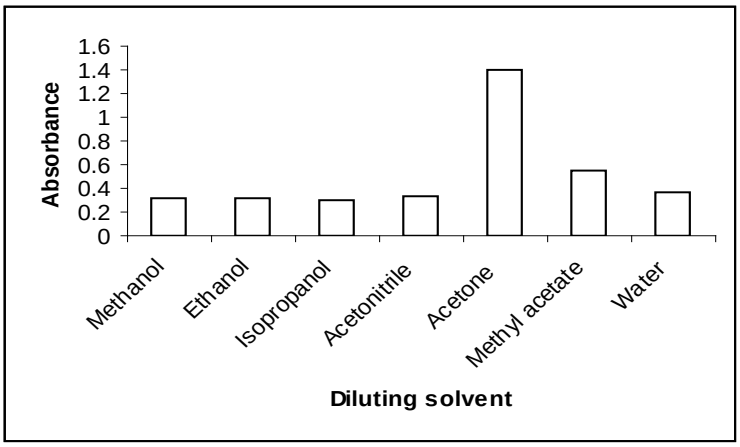

Figure 4.4: Effect of diluting solvent on NQS method; {ALS 50µg/ml, 0.5% NQS (1ml), 0.01M NaOH (1ml)}.

4.1.2.7 Effect of temperature and time

Table 4.7 show the effect of different temperatures in different times on the reaction of NQS. The room temperature (25 °C) was satisfactory for the reaction and the reaction time was 10 min.

Table 4.7: Effect of temperature and time on NQS method.

Sample	Temperature °C	Time (min)	Absorbance[a]
1	25±3	5	0.317
2		10	0.360
3		15	0.334
4	40±3	5	0.306
5		10	0.320
6		15	0.340
7	50±3	5	0.370
8		10	0.317
9		15	0.345
10	60±3	5	0.325
11		10	0.303
12		15	0.312

[a]: values were mean of three determinations; ALS 50µg/ml, 0.5% NQS (1ml), 0.01M NaOH (1ml), H_2O diluting solvent.

4.1.2.8 Order of addition effect

Table 4.8 show the effect of different order of addition on the reaction.

Table 4.8: Order of addition effect on NQS method.

Sample	First	Second	Third	Absorbance[a]
1	NQS	NaOH	ALS	0.360
2	NQS	ALS	NaOH	0.390
3	NaOH	NQS	ALS	0.365
4	NaOH	ALS	NQS	0.358
5	ALS	NQS	NaOH	0.374
6	ALS	NaOH	NQS	0.382
No effective difference in order of addition.				

[a]: values were mean of three determinations; ALS 50µg/ml, 0.5% NQS (1ml), 0.01M NaOH (1ml), H2O diluting solvent, 10 min reaction time at room temperature.

4.1.2.9 Stability of ALS-NQS chromogen

Figure 4.5 show the effect of time on the stability of ALS-NQS chromogen after dilution. The stability of ALS-NQS chromogen remained for at least 2 hr.

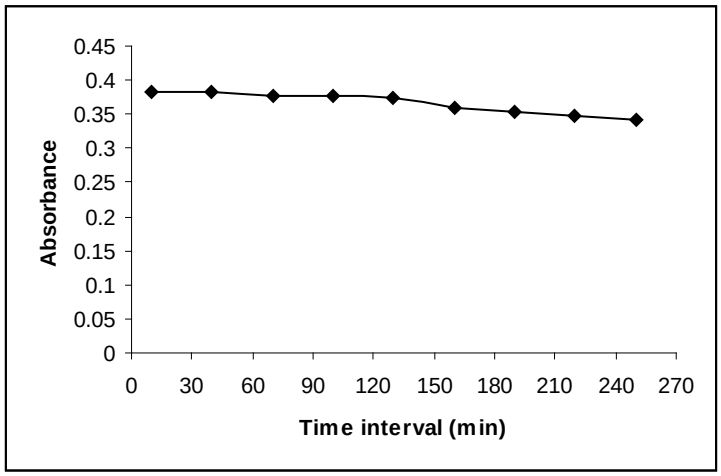

Figure 4.5: Stability of ALS-NQS chromogen; {ALS 50µg/ml, 0.5% NQS (1ml), 0.01M NaOH (1ml), H_2O diluting solvent; time intervals begun after 10 min and after dilution; continued up to 4 hours}.

4.1.2.10 Determination of stoichiometric ratio

4.1.2.10.1 Limiting logarithmic method

Figure 4.6 show the obtained lines and the slopes of these lines. The slopes for first and second sets of experiments were 1.979 and 1.0324, respectively. This confirms the 2:1 ratio for NQS and ALS reaction, respectively.

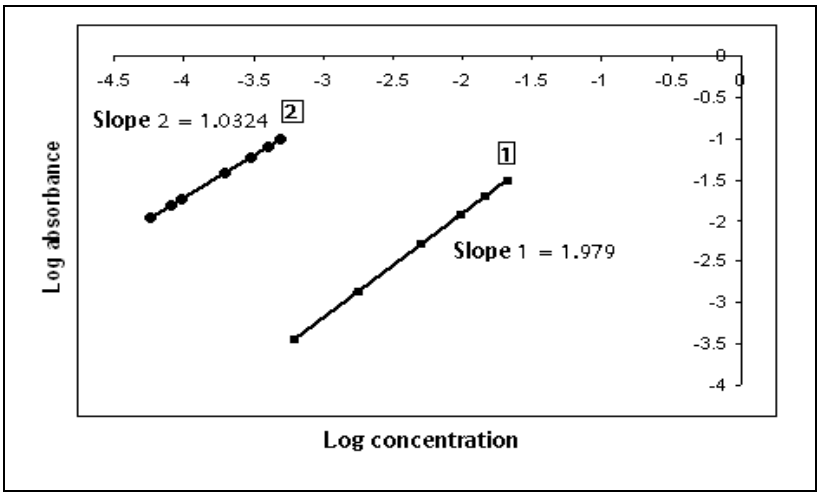

Figure 4.6: Limiting logarithmic plot for molar reactivity of ALS with NQS; {1: first set of experiments, NQS concentrations ($1.9 \times 10^{-3} – 19.2 \times 10^{-3}$ M) at ALS concentration (1.8×10^{-4} M); 2: second set of experiments, ALS concentrations ($0.4 \times 10^{-4} – 5 \times 10^{-4}$ M) at NQS concentration (1.9×10^{-2} M)}.

4.1.2.10.2 Job's method

Figure 4.7 show the mole fraction of NQS versus absorbances obtained. The mole ratio obtained for NQS and ALS was 2:1.

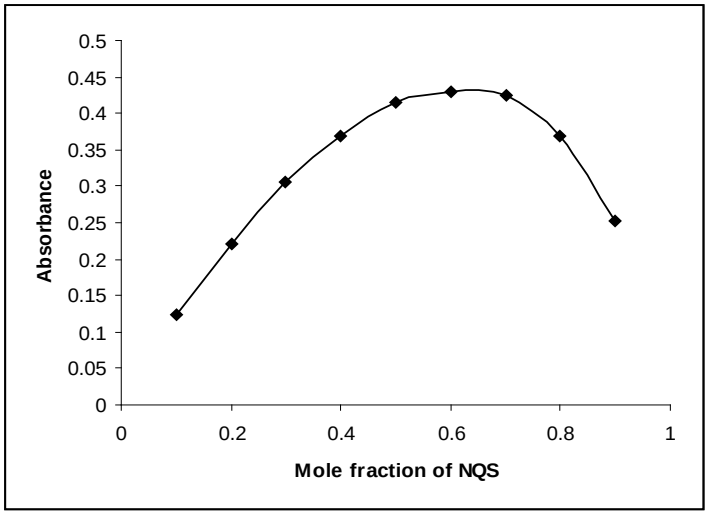

Figure 4.7: Determination of ALS-NQS chromogen ratio by Job's method.

4.1.2.11 Summary of optimization studies of NQS method

Table 4.9 show the summary for the optimization of variables affecting the reaction of ALS with NQS reagent employed in the development of the proposed spectrophotometric method.

Table 4.9: Summary of optimum conditions for ALS-NQS reaction.

Variable	Studied range	Optimum
NQS concentration (%, w/v)	0.05-1	0.5
Volume of 0.5% NQS (ml)	0.5-2	1
Inorganic bases	NaOH, $NaHCO_3$, $NaHPO_4$	NaOH
NaOH concentration (molarity)	0.01-1	0.01
Volume of 0.01 M NaOH (ml)	0.5-2	1
pH	3-14	10-11.5
Temperature (° C)	25-60	25
Time (min)	5-15	10
Diluting solvent	Different[a]	H_2O
Order of addition	Different	*
Measuring wavelength (nm)	300-700	500

[a]: solvents tested were; methanol, ethanol, isopropanol, acetonitrile, acetone, methyl acetate and water; *: no difference in order of addition was observed.

4.1.3 Validation of NQS method

Validation parameters were carried out according to ICH guidelines (*ICH, 2005*).

4.1.3.1 Linearity

Under the optimum reaction conditions (Table 4.9), the calibration curve for the determination of ALS by its reaction with NQS was constructed by plotting the absorbances as a function of the corresponding concentrations (µg/ml). Table 4.10 shows the regression equation parameters and statistical analysis. Figure 4.8 show the calibration curve.

Table 4.10: Regression equation parameters of NQS method.

Regression equation	**Intercept (a)**	**Slope (b)**	**Correlation coefficient (R^2)**
1	0.099	0.0051	0.9916
2	0.1059	0.005	0.9907
3	0.1394	0.005	0.9903
4	0.1068	0.0051	0.9909
5	0.1024	0.005	0.9901
SD	0.016338	0.0000548	0.000585
Mean ± SD	**0.1107±0.016338**	**0.00504±0.0000548**	**0.99072±0.000585**
λ_{max} (ALS-NQS chromogen) = 500 nm			
Linear range = 20-300 µg/ml			
Regression equation: Y = 0.005 X + 0.1107 (n = 5)			
Molar absorptivity (ε, L/mol.cm) = 3.25 ± 0.4722 × 10^5			
p-value of slope = 0.000			
p-value of intercept = 0.035			

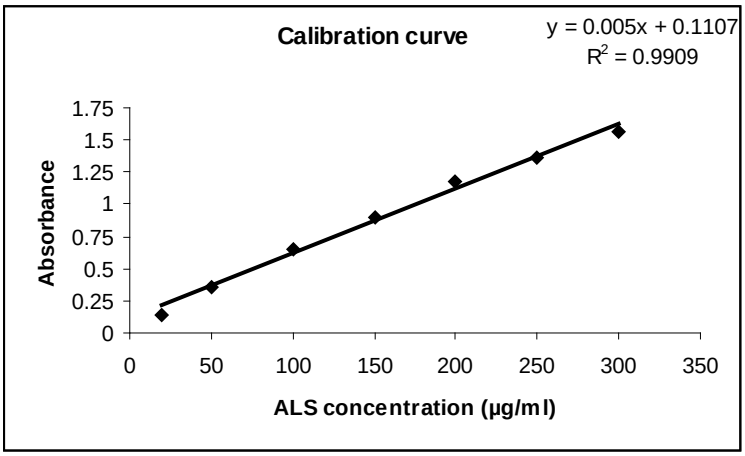

Figure 4.8: Calibration curve of NQS method; (mean of five replicates).

4.1.3.2 Range

The specified range was derived from linearity studies and it was found to be in the range of 20-300 µg/ml.

4.1.3.3 Accuracy

The accuracy of the method was evaluated by the recovery studies for added concentrations. The recovery values are 99.17 – 100.65 ± 0.28 – 1.7% (Table 4.11), indicating the accuracy of the developed method.

Table 4.11: Accuracy of NQS method.

Sample	Preanalyzed drug product (µg/ml)	ALS standard addition (µg/ml)	Actual ALS found (µg/ml)[a]	Recovery (% ± SD)[a]
1	50	25	74.75	99.17 ± 0.28
2		50	100.32	100.65 ± 0.99
3		75	124.93	99.9 ± 1.2
4	80	40	120.19	100.5 ± 0.76
5		80	159.73	99.65 ± 0.28
6		120	200.39	100.33 ± 1
7	100	50	150.26	100.52 ± 1
8		100	199.86	99.86 ± 1.7
9		150	249.53	99.68 ± 0.28

[a]: values were mean of three determinations.

4.1.3.4 Specificity

4.1.3.4.1 Identification

Identification test was achieved by comparing the measurements at the same wavelength 500 nm of ALS-NQS chromogen against blank (figure 4.9) versus blank against water (figure 4.10).

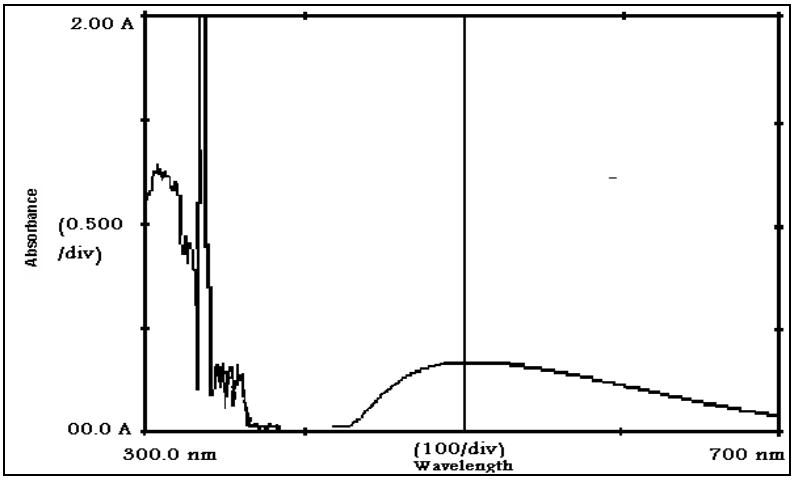

Figure 4.9: ALS-NQS chromogen spectrum; {ALS 50µg/ml}.

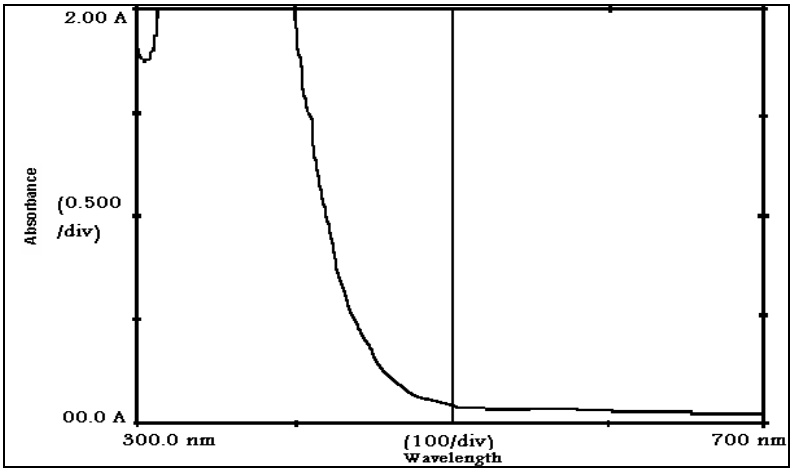

Figure 4.10: NQS blank solution spectrum; {Blank solution: 1 ml 0.5% NQS solution and 1 ml 0.01 M NaOH solution diluted up to 10 ml with distilled water}.

4.1.3.4.2 Assay

The assay of the method was demonstrated by adding appropriate levels of excipients to the drug and calculating the percent of the drug recovery. The average recovery values for 50 and 100 mg are 99.95 ± 1.8% and 100.86 ± 1%, respectively. Table 4.12 shows the interferences liabilities from inactive ingredients.

Table 4.12: Interferences liabilities from excipients on NQS method.

Sample	Excipients	ALS added (mg)	Recovery (% ± SD)[a]
1	Glucose (50)[b]	50	99.32 ± 0.15
		100	101.86 ± 0.078
2	Lactose (50)	50	98.12 ± 0.04
		100	99.86 ± 0.26
3	Starch (50)	50	101.32 ± 0.32
		100	100.86 ± 0.2
4	Talc (50)	50	101.72 ± 0.4
		100	101.46 ± 0.3
5	TiO_2[c] (50)	50	102.12 ± 0.24
		100	101.26 ± 0.5
6	Povidone (10)	50	99.72 ± 1.1
		100	101.66 ± 1.2
7	MS[d] (10)	50	97.32 ± 1
		100	99.06 ± 0.5
Average ± SD	50		99.95 ± 1.8
	100		100.86 ± 1

[a]: values were mean of three determinations; [b]: figures in parenthesis are the amounts in mg added to ALS powder; [c]: titanium dioxide; [d]: magnesium stearate.

4.1.3.5 Precision

4.1.3.5.1 Repeatability

The intra-assay precision of the method was determined on samples of drug solutions at varying concentration levels (Table 4.13) by measuring five replicates of each sample as a batch in a single assay run. The RSD did not exceed 0.62% proving the high precision of the method.

Table 4.13: Repeatability of NQS method.

Sample	ALS actual conc. (µg/ml)	ALS Found conc. (µg/ml)	Mean	SD	RSD (%)
1	50	50.06	49.88	0.29	0.581
2		49.66			
3		49.86			
4		49.56			
5		50.26			
1	150	149.86	150.46	0.93	0.618
2		151.86			
3		150.86			
4		150.26			
5		149.46			
1	250	249.86	249.82	0.86	0.344
2		250.46			
3		250.86			
4		249.06			
5		248.86			

4.1.3.5.2 Intermediate precision

The inter-assay precision of the method was determined on samples of drug solutions at varying concentration levels (Table 4.14) by analyzing each sample as a batch in a single assay run for six consecutive days. The RSD did not exceed 1.7% proving good intermediate precision of the method.

Table 4.14: Intermediate precision of NQS method.

Sample	ALS actual conc. (µg/ml)	ALS found conc. (µg/ml)						Mean	SD	RSD (%)
1	50	49.86	50.86	48.66	49.06	50.66	49.86	49.83	0.86	1.7
2	150	149.86	149.06	150.86	149.26	149.66	149.86	149.76	0.63	0.421
3	250	249.86	250.46	250.86	249.06	249.26	250.06	249.93	0.69	0.276

4.1.3.6 LOD

The detection limit was 1.6 µg/ml based on the residual standard deviation of the regression line (0.002425) and the slope (0.005) (LOD = 3.3 σ / S).

4.1.3.7 LOQ

The quantitation limit was 4.85 µg/ml based on the residual standard deviation of the regression line (0.002425) and the slope (0.005) (LOQ = 10 σ / S).

4.1.3.8 Robustness

Robustness was examined by evaluating the influence of small variation in the method variables on its analytical performance. It was found that small variation in the method variables did not significantly affect the procedures; recovery values were 98.2 – 102.78 ± 0.14 – 1.6% (Table 4.15). This indicated the reliability of the method.

Table 4.15: Robustness of NQS method.

Parameter	Normal	Variation	Recovery (% ± SD)[a]
NQS concentration (% w/v)	0.5	0.4	99.97 ± 0.33
		0.6	99.2 ± 1.5
0.5% NQS volume (ml)	1	0.8	101.5 ± 1.5
		1.2	100.4 ± 0.68
NaOH Molarity (M)	0.01	0.008	99.92 ± 1.6
		0.012	98.2 ± 0.52
0.01 M NaOH volume (ml)	1	0.8	98.67 ± 0.48
		1.2	102.78 ± 1.3
Temperature of the reaction (°C)	25	20	98.9 ± 0.4
		30	99.44 ± 0.24
Time of the reaction (min)	10	8	99.28 ± 0.14
		12	100.65 ± 0.39

[a]: values were mean of three determinations; The concentration of ALS was 50µg/ml.

4.1.3.9 Ruggedness

Ruggedness was tested by applying the procedure using the same operational conditions but using two different instruments at two different laboratories and different elapsed time. Results obtained from lab-to-lab variations were reproducible, as RSD did not exceed 0.7% (Table 4.16).

Table 4.16: Ruggedness of NQS method.

Lab.	ALS actual conc. (µg/ml)	ALS found conc. (µg/ml)			Mean	SD	RSD (%)
1	50	50.46	49.86	50.46	50.26	0.35	0.7
	150	149.86	150.26	150.26	150.13	0.23	0.15
	250	249.66	250.26	249.26	249.73	0.5	0.2
2	50	50.26	50.26	49.66	50.06	0.35	0.7
	150	149.86	150.26	150.06	150.06	0.2	0.13
	250	249.86	249.26	249.06	249.4	0.42	0.17

4.1.4 Analysis of commercial pharmaceutical product

The pharmaceutical dosage form was subjected to the analysis of their ALS content by NQS method and reference method *(Wrasse-Sangoi et al, 2010)*. The recovery percentage was 99.76 ± 0.57 (Table 4.17). This result was compared with that obtained from reference method by statistical analysis with respect to the accuracy by *t*-test (Table 4.18). No significant difference was found at 95% confidence level providing similar accuracy in the determination of ALS by both methods.

Table 4.17: Determination of ALS in tablets by NQS and reference method.

Tablet[a]	Recovery (% ± SD)[b]	
	NQS method	Reference method[c]
	99.76 ± 0.57	100.76 ± 0.83[d]
Label claim[b]	299.28 ± 0.57 mg	302.28 ± 0.83 mg

[a]: labeled to contain 300 mg ALS per tablet; [b]: Values were mean of five determinations;
[c]: Reference method: (*Wrasse-Sangoi et al, 2010*); [d]: Calculated in our lab.

Table 4.18: (t-test) between NQS and reference method.

NQS and reference	t-value	d_f	Significant (2-tailed)	Mean Difference	Standard Error Difference	95% Confidence level of difference	
						Lower	Upper
Concentration	2.212	8	0.058	0.50800	0.22970	1.03770	0.02170

4.2 Method II (NIN derivatization method)

4.2.1 Selection of analytical wavelength of ALS-NIN chromogen

The absorption spectra and maximum absorption point of the ALS-NIN chromogens of 50 and 100 µg/ml for ALS as final concentrations (figure 4.11) were determined against blank solution on the UV/Vis spectrophotometer. The maximum absorption point was 569 nm.

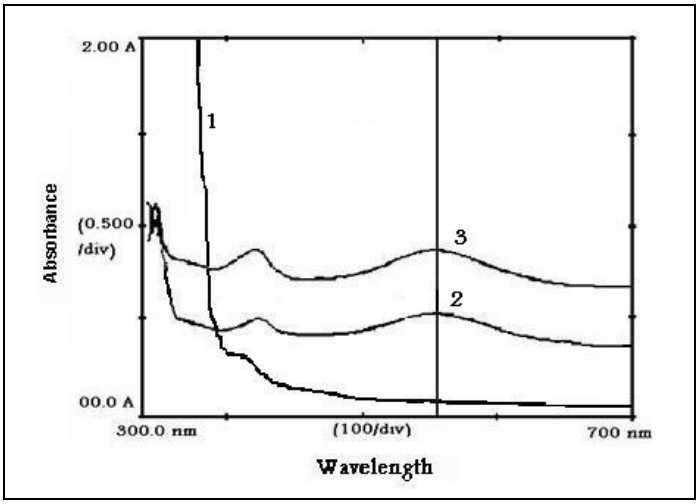

Figure 4.11: Selection of analytical wavelength of ALS-NIN chromogen, {1: blank spectrum against water; 2 and 3: chromogen against blank at ALS 50 and 100 µg/ml}.

4.2.2 Optimization of NIN reaction conditions

4.2.2.1 Effect of NIN concentration

Table 4.19 and figure 4.12 show the effect of NIN concentration% on the derivatization reaction. The maximum absorption was obtained with 1.2% NIN solution.

Table 4.19: Effect of NIN concentration on NIN method.

Sample	NIN concentration % (w/v, 1ml)	Absorbance[a]
1	0.7	0.0981
2	0.8	0.17195
3	0.9	0.22665
4	1	0.27765
5	1.1	0.2912
6	1.2	0.30855
7	1.3	0.2101
8	1.5	0.14315
9	2	0.079

[a]: values were mean of three determinations; {ALS 100µg/ml, 0.2M phosphate buffer pH 6.0 (1ml), 90 °C reaction temperature and 20 min reaction time}.

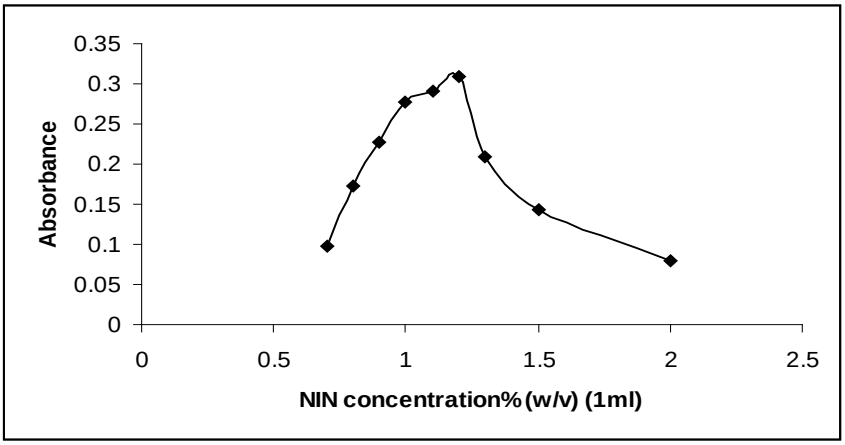

Figure 4.12: Effect of NIN concentration on NIN method; {ALS 100µg/ml, 0.2M phosphate buffer pH 6.0 (1ml), 90 °C reaction temperature and 20 min reaction time}.

4.2.2.2 Effect of 1.2% NIN solution volume

Table 4.20 show the effect of volume of 1.2% NIN solution on the reaction. The best volume was 0.5 ml.

Table 4.20: Effect of 1.2% NIN volume on NIN method.

Sample	1.2% NIN solution volume (ml)	Absorbance[a]
1	0.5	0.4626
2	1	0.30855
3	1.5	0.27545
4	2	0.2218

[a]: values were mean of three determinations; {ALS 100µg/ml, 0.2M of phosphate buffer pH 6.0 (1ml), 90 °C reaction temperature and 20 min reaction time}.

4.2.2.3 Effect of buffer system type

The effect of buffer system type was investigated by using different types of buffer systems. We investigated phosphate and citrate buffer systems adjusted to pH 6.0 prepared in 0.2M concentrations (Table 4.21). Best result was obtained with 0.2 M phosphate buffer.

Table 4.21: Effect of buffer system type on NIN method.

Sample	Type of buffer system (1ml)	Absorbance[a]
1	0.2M phosphate buffer pH 6.0	0.4626
2	0.2M citrate buffer pH 6.0	0.31845

[a]: values were mean of three determinations; {ALS 100µg/ml, 0.5ml 1.2% NIN solution, 90 °C reaction temperature and 20 min reaction time}.

4.2.2.4 Effect of pH

The influence of pH on the absorbance of the produced chromogen was investigated by carrying out the reaction in buffer solution at various pH values of 0.2M citrate buffer system (4.5, 5, 5.5, 6, and 6.5) and 0.2 M phosphate buffer system (6, 6.5, and 7) (Table 4.22). Best result was obtained with 0.2 M phosphate buffer system pH 6.0.

Table 4.22: Effect of pH on NIN method.

Sample	**pH of 0.2M of citrate buffer solution (1ml)**	**Absorbance[a]**
1	4.5	0.042
2	5	0.177
3	5.5	0.2785
4	6	0.3185
5	6.5	0.375
Sample	**pH of 0.2M of phosphate buffer solution (1ml)**	**Absorbance[a]**
6	6	0.4626
7	6.5	0.4162
8	7	0.4104

[a]: values were mean of three determinations; {ALS 100µg/ml, 0.5ml 1.2% NIN solution, 90 °C reaction temperature and 20 min reaction time }.

4.2.2.5 Effect of volume of 0.2 M phosphate buffer pH 6.0

Table 4.23 show the effect of volume of 0.2 M phosphate buffer pH 6.0. The best volume was 1 ml.

Table 4.23: Effect of volume of 0.2M phosphate buffer pH 6.0 on NIN method.

Sample	**Volume of 0.2M of phosphate buffer pH 6.0 (ml)**	**Absorbance[a]**
1	0.5	0.4397
2	1	0.4626
3	1.5	0.3126
4	2	0.1686

[a]: values were mean of three determinations; {ALS 100µg/ml, 0.5ml 1.2% NIN solution, 90 °C reaction temperature and 20 min reaction time}.

4.2.2.6 Effect of type and concentration% of reducing agent

Different reducing agents were tested with different concentration% prepared as aqueous solutions: ascorbic acid, sodium molybdate and zinc chloride. Table 4.24 shows the effect of type and concentration% of reducing agents.

Table 4.24: Effect of type and concentration% of reducing agents on NIN method.

Sample	Ascorbic acid% (w/v, 1ml)	Absorbance[a]
1	0.1	0.341
2	0.2	0.319
3	1	*
Sample	**Sodium molybdate% (w/v, 1ml)**	**Absorbance[a]**
1	0.1	0.03
2	0.2	0.04
3	1	0.06
Sample	**Zinc chloride% (w/v, 1ml)**	**Absorbance[a]**
1	0.1	0.0687
2	0.2	0.0705
3	1	0.0992

[a]: values were mean of three determinations, *: yellow color, indicate that the ascorbic acid reacted with ALS and no absorption at 569 nm was found; {ALS 100µg/ml, 0.5ml of 1.2% of NIN solution, 1ml of 0.2M phosphate buffer pH 6.0, 90 °C reaction temperature and 20 min reaction time}.

The use of reducing agents lead to decrease of absorbance of produced chromogen. In subsequent experiments reducing agents were excluded.

4.2.2.7 Effect of diluting solvent

Table 4.25 and figure 4.13 show the effect of using different solvents in the final dilution of the reaction. Water was the best solvent for dilution.

Table 4.25: Effect of diluting solvent on NIN method.

Sample	Diluting solvent	Absorbance[a]
1	Methanol	0.42
2	Ethanol	0.248
3	Isopropanol	0.15
4	Acetonitrile	0.313
5	Ethyl acetate	*
6	Water	0.4626

[a]: values were mean of three determinations, *: the chromogen did not dissolve in the solvent; {ALS 100µg/ml, 0.5ml 1.2% of NIN solution, 1ml 0.2M of phosphate buffer pH 6.0, 90 °C reaction temperature and 20 min reaction time}.

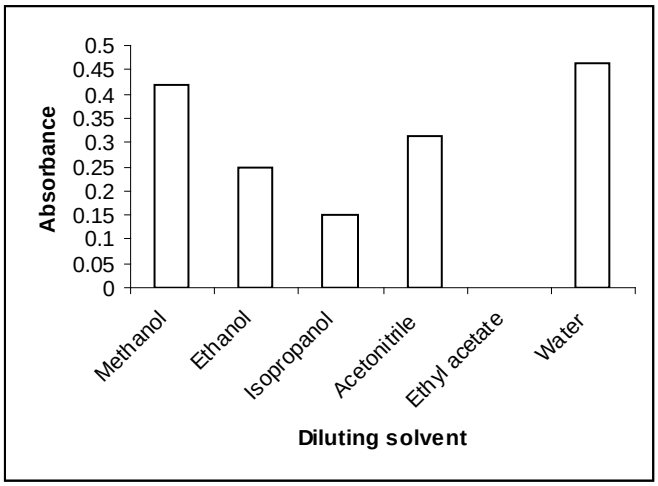

Figure 4.13: Effect of diluting solvent on NIN method; {ALS 100µg/ml, 0.5ml 1.2% of NIN solution, 1ml 0.2M phosphate buffer pH 6.0, 90 °C reaction temperature and 20 min reaction time}.

4.2.2.8 Effect of temperature and time

Table 4.26 shows the effect of different temperatures in different time on the reaction of NIN and the importance of heating. Maximum absorbance was obtained in case of 90 °C for 20 min.

Table 4.26: Effect of temperature and time on NIN method.

Sample	Temperature (°C)	Time (min)	Absorbance[a]
1	80±3	5	0.12915
2		10	0.1667
3		15	0.24585
4		20	0.31665
5		25	0.3345
1	90±3	5	0.16125
2		10	0.31365
3		15	0.46765
4		20	0.50835
5		25	0.39955
1	100±3	5	0.11125
2		10	0.4626
3		15	0.4751
4		20	0.4781
5		25	0.4814
No color developed up to 4 hours at room temperature.			

[a]: values were mean of three determinations; {ALS 100µg/ml, 0.5ml 1.2% of NIN solution, 1ml 0.2M phosphate buffer pH 6.0}.

4.2.2.9 Order of addition effect

Table 4.27 summarizes the studied combination solutions on the reaction of ALS with NIN. Table 4.28 show the effect of volume of N/A/PH solution on the reaction.

Table 4.27: Order of addition effect on NIN method.

Number	Combination solution and volume (ml)	Beer's law limits (µg/ml)	Correlation coefficient (R^2)
1	1.2% NIN in phosphate buffer pH 6.0 (1ml)	50-250	0.96[a]
2	1.2% NIN in phosphate buffer pH 6.0 (1ml) + 0.1% ascorbic acid in water (1ml)	50-200	0.98[a]
3	1.2% NIN + 0.1% ascorbic acid in 0.2 M phosphate buffer pH 6.0 (N/A/PH) (1ml)	10-170	0.992[b]
4	1.2% NIN + 0.1% ascorbic acid in water (1ml) + 0.2 M phosphate buffer pH 6.0 (0.5ml)	50-200	0.997[a]
5	1.2% NIN + 0.03% ascorbic acid in 0.2 M phosphate buffer pH 6.0 (1ml)	10-80	0.997[a]

[a]: values were mean of three determination; [b]: Values were mean of five determinations.

Table 4.28: Effect of volume of N/A/PH solution on NIN method.

Sample	Volume of N/A/PH solution (ml)	Absorbance[a]
1	0.5	0.737
2	1	0.97
3	1.5	1.246
4	2	1.382

a: values were mean of three determinations; {ALS 100µg/ml, temperature 90 °C and reaction time 20 min}.

1 ml of N/A/PH was used in the calibration curve and for validation parameters.

4.2.2.10 Stability of ALS-NIN chromogen

Figure 4.14 show the effect of time on the stability of ALS-NIN chromogen after dilution. The stability of ALS-NIN chromogen remained for at least 1.5 hr.

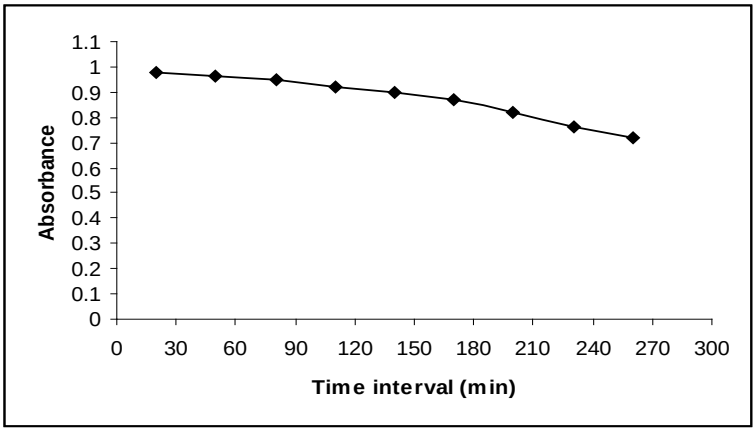

Figure 4.14: Stability of ALS-NIN chromogen; {ALS 100µg/ml, 1ml N/A/PH solution, temperature was 90 °C}.

4.2.2.11 Determination of stoichiometric ratio

4.2.2.11.1 Limiting logarithmic method

Figure 4.15 show the obtained lines and the slopes of these lines. The slopes for first and second sets of experiments were 2.053 and 1.0828, respectively. This confirms the 2:1 ratio for NIN and ALS reaction, respectively.

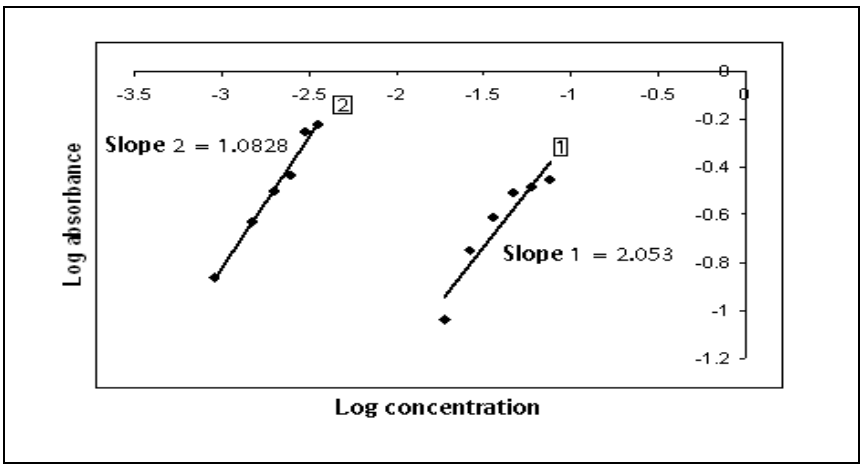

Figure 4.15: Limiting logarithmic plot for molar reactivity of ALS with NIN; {1: first set of experiments, NIN concentrations ($3.9 \times 10^{-3} – 11 \times 10^{-3}$ M) at ALS concentration (1.8×10^{-4} M); 2: second set of experiments, ALS concentrations ($0.9 \times 10^{-4} – 3.6 \times 10^{-4}$ M) at NIN concentration (6.7×10^{-3} M)}.

4.2.2.11.2 Job's method

Figure 4.16 show the mole fraction of NIN versus absorbances obtained. The mole ratio obtained for NIN and ALS was 2:1.

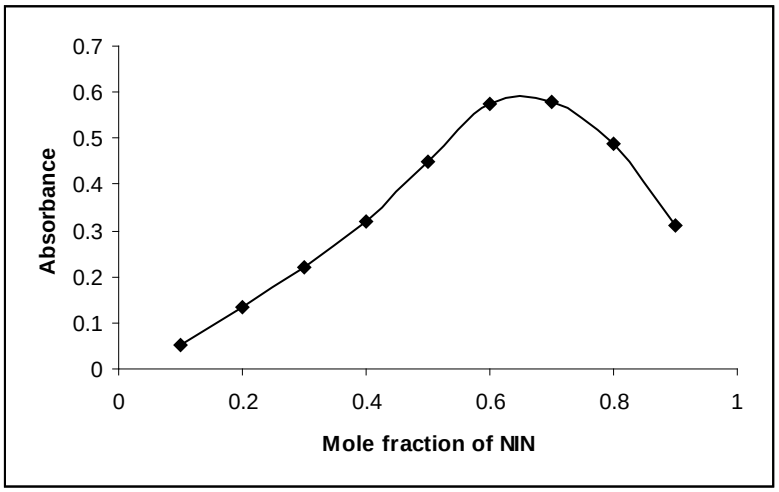

Figure 4.16: Determination of ALS-NIN chromogen ratio by Job's method.

4.2.2.12 Summary of optimization studies of NIN method

Table 4.29 show the summary for the optimization of variables affecting the reaction of ALS with NIN reagent employed in the development of the proposed spectrophotometric method.

Table 4.29: Summary of optimum conditions for ALS-NIN reaction.

Variable	Studied range	Optimum
NIN concentration% (w/v)	0.7-2	1.2
Volume of 1.2% NIN solution (ml)	0.5-2	0.5
Buffer system type at pH 6.0	0.2M citrate and 0.2M phosphate buffers	0.2M phosphate buffer
pH	4.5-7	6.0
Volume of 0.2M phosphate buffer pH 6.0 (ml)	0.5-2	1
Type and concentration% of reducing agents	Ascorbic acid, sodium molybdate, $ZnCl_2$ (0.1, 0.2 and 1% w/v)	*
Diluting solvent	Different[a]	H_2O
Temperature (°C)	80-100	90
Time (min)	5-25	20
Order of addition	Different combination solutions[b]	N/A/PH
Volume of N/A/PH solution (ml)	0.5-2	1
Measuring wavelength (nm)	400-700	569

*: absorption decreased with reducing agents; [a]: solvents tested were: methanol, ethanol, isopropanol, acetonitrile, ethyl acetate and water; [b]: combination solutions were: 1.2% NIN in 0.2 M phosphate buffer pH 6.0, 1.2% NIN in 0.2 M phosphate buffer pH 6.0 + 0.1% ascorbic acid in water, 1.2% NIN + 0.1% ascorbic acid in 0.2 M phosphate buffer pH 6.0 (N/A/PH), 1.2% NIN + 0.1% ascorbic acid in water + 0.2M phosphate buffer pH 6.0 and 1.2% NIN + 0.03% ascorbic acid in 0.2 M phosphate buffer pH 6.0.

4.2.3 Validation of NIN method

Validation parameters were carried out according to ICH guidelines (*ICH, 2005*).

4.2.3.1 Linearity

Under the optimum reaction conditions (Table 4.29), the calibration curve for the determination of ALS by its reaction with NIN was constructed by plotting the absorbances as a function of the corresponding concentrations (µg/ml). Table 4.30 shows the regression equation parameters and statistical analysis. Figure 4.17 show the calibration curve.

Table 4.30: Regression equation parameters of NIN method.

Regression equation	**Intercept (a)**	**Slope (b)**	**Correlation coefficient (R^2)**
1	0.1638	0.0083	0.9913
2	0.1778	0.0081	0.99
3	0.1535	0.0085	0.991
4	0.1553	0.0083	0.9933
5	0.159	0.008	0.991
SD	0.0097	0.000195	0.0012
Mean ± SD	**0.16188±0.0097**	**0.0082±0.000195**	**0.991±0.0012**
λ_{max} (ALS-NIN chromogen) = 569 nm			
Linear range = 10-170 µg/ml			
Regression equation: Y = 0.008 X + 0.161 (n = 5)			
Molar absorptivity (ε, L/mol.cm) = 5.3 ± 0.50215 × 10^5			
p-value of slope = 0.000			
p-value of intercept = 0.04			

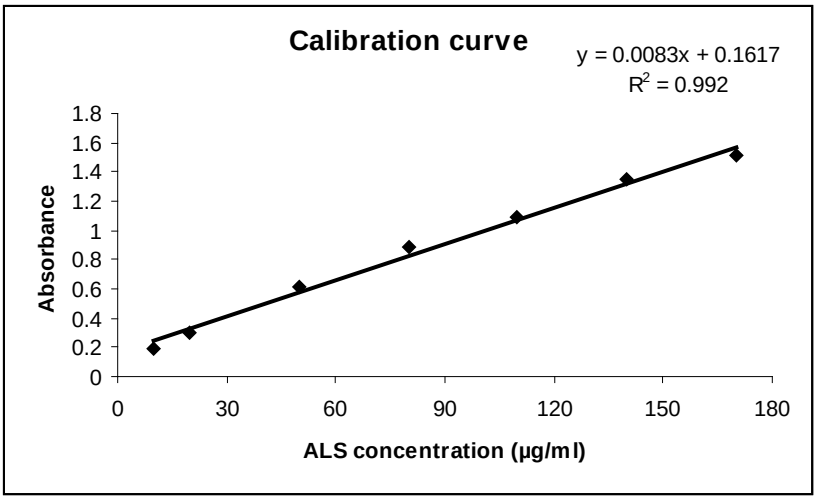

Figure 4.17: Calibration curve of NIN method; (mean of five replicates).

4.2.3.2 Range

The specified range was derived from linearity studies and it was found to be in the range of 10-170 µg/ml.

4.2.3.3 Accuracy

The accuracy of the method was evaluated by the recovery studies for added concentrations. The recovery values are 99.63 – 101.2 ± 0.25 – 1.27% (Table 4.31), indicating the accuracy of the developed method.

Table 4.31: Accuracy of NIN method.

Sample	**Preanalyzed drug product (µg/ml)**	**ALS standard addition (µg/ml)**	**Actual ALS found (µg/ml)[a]**	**Recovery (% ± SD)[a]**
1	20	10	30.36	101.2 ± 0.98
2		20	40.34	100.85 ± 0.64
3		30	50.17	100.34 ± 0.38
4	40	20	60.54	100.9 ± 0.63
5		40	80.58	100.7 ± 0.62
6		60	99.63	99.63 ± 0.25
7	60	30	91.1	101.2 ± 1.27
8		60	119.9	99.9 ± 0.31
9		90	150.17	100.13 ± 0.73

[a]: values were mean of three determinations.

4.2.3.4 Specificity

4.2.3.4.1 Identification

Identification test was achieved by comparing the measurements at the same wavelength 569 nm of ALS-NIN chromogen against blank (figure 4.18) versus blank against water (figure 4.19).

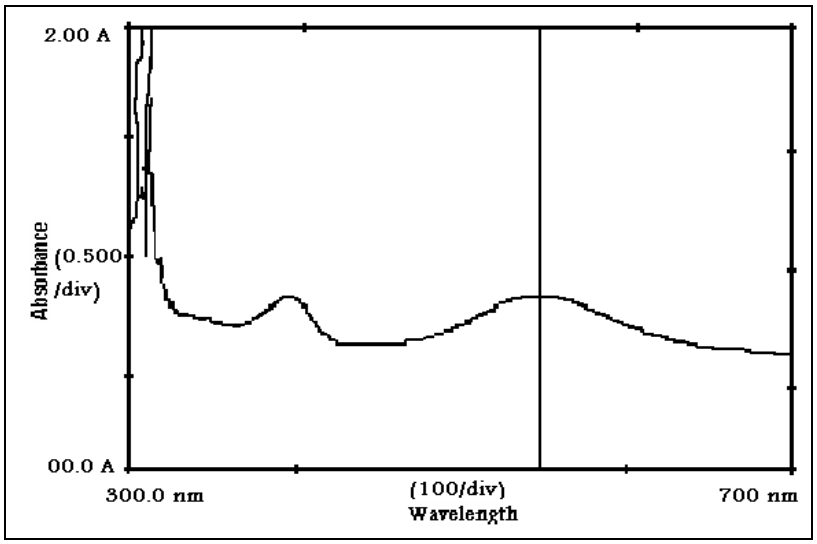

Figure 4.18: ALS-NIN chromogen spectrum; {ALS 100µg/ml}.

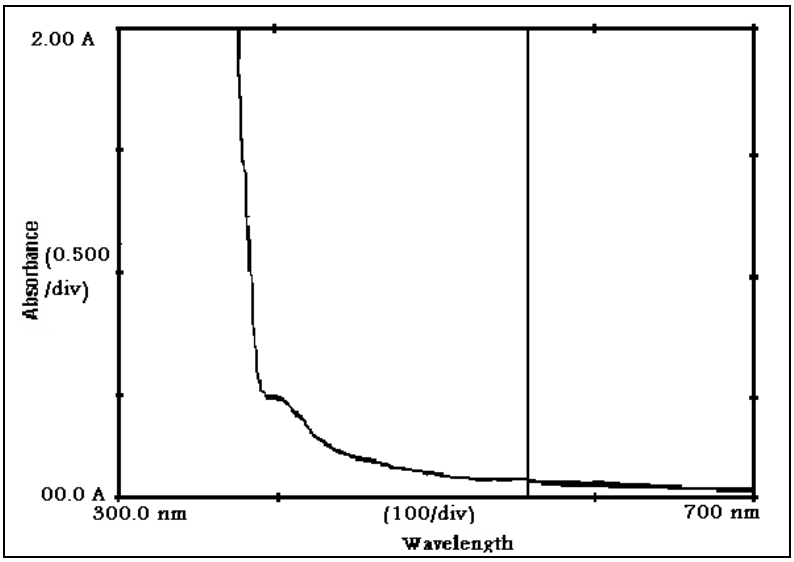

Figure 4.19: NIN blank solution spectrum; {Blank solution was 1 ml N/A/PH solution diluted up to 10 ml with distilled water}.

4.2.3.4.2 Assay

The assay of the method was demonstrated by adding appropriate levels of excipients to the drug and calculating the percent of the drug recovery. The average recovery values for 50 and 100 mg are 99.13 ± 0.76% and 100.3 ± 0.82%, respectively. Table 4.32 shows the interferences liabilities from inactive ingredients.

Table 4.32: Interferences liabilities from excipients on NIN method.

Sample	Excipients	ALS powder added (mg)	Recovery (% ± SD)[a]
1	Glucose (50)[b]	50	99.21 ± 0.31
		100	100.4 ± 0.73
2	Lactose (50)	50	98.9 ± 1.21
		100	99.6 ± 0.58
3	Starch (50)	50	98.7 ± 0.93
		100	99.5 ± 1.03
4	Talc (50)	50	99.25 ± 0.45
		100	101.3 ± 0.75
5	TiO_2[c] (50)	50	98.8 ± 0.69
		100	100.5 ± 0.27
6	Povidone (10)	50	99.61 ± 0.83
		100	101.2 ± 0.48
7	MS[d] (10)	50	99.43 ± 0.92
		100	100.3 ± 0.82
Average ± SD		50	99.13 ± 0.76
		100	100.4 ± 0.66

[a]: values were mean of three determinations; [b]: figures in parenthesis are the amounts in mg added to ALS powder; [c]: titanium dioxide; [d]: magnesium stearate.

4.2.3.5 Precision

4.2.3.5.1 Repeatability

The intra-assay precision of the method was determined on samples of drug solutions at different concentration levels (Table 4.33) by measuring five replicates of each sample as a batch in a single assay run. RSD did not exceed 1.6% proving the good precision of the method.

Table 4.33: Repeatability of NIN method.

Sample	ALS actual conc. (µg/ml)	ALS found conc. (µg/ml)	Mean	SD	RSD (%)
1	20	19.75	20.025	0.32	1.6
2		20.375			
3		19.625			
4		20.25			
5		20.125			
1	80	81	79.9	0.91	1.13
2		79.87			
3		80.87			
4		79.25			
5		79			
1	140	139.625	140.075	0.58	0.414
2		140.5			
3		140.125			
4		140.75			
5		139.375			

4.2.3.5.2 Intermediate precision

The inter-assay precision of the method was determined on samples of drug solutions at varying concentration levels (Table 4.34) by analyzing each sample as a batch in a single assay run for six consecutive days. The RSD did not exceed 1.46% proving good intermediate precision of the method.

Table 4.34: Intermediate precision of NIN method.

Sample	ALS actual conc. (µg/ml)	ALS found conc. (µg/ml)						Mean	SD	RSD (%)
1	20	19.625	20.5	19.375	19.75	20.125	20	19.89	0.29	1.46
2	80	81	79.87	80.87	79.25	79	79.87	79.98	0.82	1.02
3	140	139.625	140.5	140.125	140.75	139.375	139.25	139.94	0.62	0.443

4.2.3.6 LOD

The detection limit was 1.27 µg/ml based on the residual standard deviation of the regression line (0.00308) and the slope (0.008) (LOD = 3.3 σ / S).

4.2.3.7 LOQ

The quantitation limit was 3.85 µg/ml based on the residual standard deviation of the regression line (0.00308) and the slope (0.008) (LOQ = 10 σ / S).

4.2.3.8 Robustness

Robustness was examined by evaluating the influence of small variation in the method variables on its analytical performance. It was found that small variation in the method variables did not significantly affect the procedures; recovery values were 98.6 – 101.1 ± 0.31 – 1.12% (Table 4.35). This indicated the reliability of the method.

Table 4.35: Robustness of NIN method.

Parameter	Normal	Variation	Recovery (% ± SD)[a]
N/A/PH	1.2% NIN	1.18%	99.6 ± 0.41
		1.22%	98.9 ± 0.64
	0.1% A.A	0.09%	101.1 ± 0.93
		0.11%	100.5 ± 0.76
	pH 6.0	5.8	98.6 ± 1.12
		6.2	99.3 ± 0.31
Temperature of the reaction (°C)	90	85	100.2 ± 0.86
		95	99.1 ± 0.37
Time of the reaction (min)	20	17	98.7 ± 0.54
		23	99.2 ± 0.62

[a]: values were mean of three determinations; The concentration of ALS was 100µg/ml.

4.2.3.9 Ruggedness

Ruggedness was tested by applying the procedure using the same operational conditions but using two different instruments at two different laboratories and different elapsed time. Results obtained from lab-to-lab variations were reproducible, as RSD did not exceed 1.85% (Table 4.36).

Table 4.36: Ruggedness of NIN method.

Lab.	ALS actual conc. (µg/ml)	ALS found conc. (µg/ml)			Mean	SD	RSD (%)
1	20	19.75	20.25	20.375	20.125	0.33	1.64
	80	79.87	79.25	79.87	79.66	0.36	0.452
	140	139.625	140.125	139.375	139.71	0.38	0.272
2	20	20.5	20.25	21	20.58	0.38	1.846
	80	81	80.87	79	80.29	1.12	1.395
	140	140.5	140.75	139.25	140.17	0.8	0.571

4.2.4 Analysis of commercial pharmaceutical product

The pharmaceutical dosage form was subjected to the analysis of their ALS content by NIN method and reference method (*Wrasse-Sangoi et al, 2010*). The recovery percentage was 100.15 ± 1.29 (Table 4.37). This result was compared with that obtained from reference method by statistical analysis with respect to the accuracy by *t*-test (Table 4.38). No significant difference was found at 95% confidence level providing similar accuracy in the determination of ALS by both methods.

Table 4.37: Determination of ALS in tablets by NIN and reference method.

Tablet[a]	Recovery (% ± SD)[b]	
	NIN method	Reference method[c]
	100.15 ± 1.29	100.76 ± 0.83[d]
Label claim[b]	300.45 ± 1.29 mg	302.28 ± 0.83 mg

[a]: labeled to contain 300 mg ALS per tablet; [b]: Values are mean of five determinations;
[c]: Reference method: (*Wrasse-Sangoi et al, 2010*); [d]: Calculated in our lab.

Table 4.38: (t-test) between NIN and reference method.

NIN and reference	t-value	d_f	Significant (2-tailed)	Mean Difference	Standard Error Difference	95% Confidence level of difference	
						Lower	Upper
Concentration	0.903	8	0.393	0.31300	0.34657	0.48620	1.11220

4.3 Comparison between NQS, NIN and reference method

Tables 4.39 and 4.40 show the comparison and the analysis of variance test (One way ANOVA) between the three methods, respectively. No significant difference was found between methods, providing similar precision in the determination of ALS content.

Table 4.39: Comparison between NQS, NIN and reference method.

Parameter	NQS method	NIN method	Reference method[a]
Beer's law limits (µg/ml)	20-300	10-170	40-100
Measured wavelength(λ_{max} nm)	500	569	279
Correlation coefficient (r^2)	0.991	0.992	0.9997
Molar absorptivity (ε, l/mol.cm)	3.25×10^5	5.3×10^5	3.05×10^{3b}
LOD (µg/ml)	1.6	1.27	2.55
LOQ (µg/ml)	4.85	3.85	8.49

[a]: Reference method (*Wrasse-Sangoi et al, 2010*); [b]: Calculated in our lab.

Table 4.40: One way ANOVA test between NQS, NIN and reference method.

Source of variation	Sum of Squares	d_f	Mean Square	F-value	Significant
Between Groups	0.657	2	0.328	1.443	0.274
Within Groups	2.730	12	0.228		
Total	3.387	14			

Chapter 5

DISCUSSION

Spectrophotometric analysis continues to be one of the most widely used analytical techniques available. Many methods are available for a variety of analytes (such as colored, colorless, natural, synthetic, inorganic and organic analytes) and sample types ranging from *in-situ* biological assays to the determination of trace elements in steels. Many medical diagnostic test kits use photometric measurements. In the food industry, wine makers have long recognized the effect of iron levels on the taste of wines and consequently are the largest users of 1,10-phenanthroline for determining iron spectrophotometrically. A common field test for chlorine in swimming pools and drinking water is based on the color produced by the action of chlorine on *o*-tolidine *(Harvey, 2000).*

The absorption spectrum of ALS was recorded against water (in our lab). It was found that ALS exhibits λ_{max} at 279 nm and the molar absorptivity (ε) was 3.05×10^3 L/mol.cm. Because of the blue-shifted λ_{max} of ALS, it's determination in the pharmaceutical formulations based on the direct measurement of its absorption for UV light is susceptible to potential interferences from the co-extracted excipients. Therefore, derivatization of ALS to a more red-shifted derivative was necessary. ALS contains primary amino group for which many chromogenic reagents are available for color-producing reactions. The choice of a robust, simple and efficient derivatization reaction is the first and most critical step for a successful and reproducibly applicable spectrophotometric method. It was attained to derivatize ALS using NQS and NIN in a single-step reaction, which is rapid, clean and accomplished. The method requires neither vigorous derivatization conditions nor any clean-up step prior to detection. The weak or lack of chromophore and the current requirement for favorable detection limits necessitated derivatization techniques for determination of ALS in spectrophotometric method. The derivatization reactions between ALS; NQS and ALS; NIN were performed and the absorption spectra of the reaction products were recorded against reagents blank. The products chromogens were orange-red colored and blue-violet colored exhibiting λ_{max} at 500 and 569 nm, respectively. Obviously, the λ_{max} of products chromogens were red-shifted from the underivatized ALS by 221 and 290 nm. As well, the values of ε, which indicate the sensitivity, were greatly enhanced to be 3.25×10^5 and 5.3×10^5 L/mol.cm, respectively. Moreover, literature survey revealed that there is no official analytical method for analysis of ALS in bulk and

pharmaceutical dosage form and the major reported analytical methods were by chromatographic technique, which is more complex, generate large amounts of waste and is considered as more time-consuming.

5.1 NQS derivatization method

Derivatization using NQS has attracted considerable attention for quantitative analysis of many pharmaceutically active compounds. The present method was based on the nucleophilic substitution reaction between ALS and NQS. The NQS reagent reacts with ALS at the free NH_2 group in alkaline conditions producing a colored chromogen which was measured at 500 nm.

Optimization of the reaction conditions was intended to take into account the various goals of the method development. Analytical conditions were optimized via a number of preliminary experiments. Studying the effect of NQS concentration and volume on its reaction with ALS revealed that the reaction was dependent on the NQS concentration as the absorbances increased with the increase in the reagent concentration. The highest absorbances were attained at a concentration range of 0.4-0.6% (w/v) (1ml) beyond which the absorbances slightly decreased. Absorption decreased with high NQS concentration may be referred to reaction saturation, as well as decreased with higher volume due to dilution of the reaction. To activate the nucleophilic substitution reaction and to generate the nucleophiles from ALS, alkaline medium was necessary. Different inorganic bases were investigated; NaOH, sodium bicarbonate and sodium hydrogen phosphate, all prepared as aqueous solution of 0.01–1 M concentration range. The influence of pH on the reaction was also investigated by carrying out the reaction in 0.1 M phosphate buffer solution of varying pH values (3-13.7). The results revealed that ALS has difficulty to react with NQS in pH < 6 (acidic media). This was possibly due to the existence of the amino group of ALS in the form of hydrochloride salt, thus it loses its nucleophilic substitution affinity. As the pH increased, the absorbances increased rapidly, as the amino group of ALS turns into the free amino group, thus facilitating the nucleophilic substitution. The maximum absorbances were attained at pH ranged from 10–11.5. At higher pH, sharp decrease in the absorbances occurred. This was attributed probably to the increase in the amount of hydroxide ion that holds back the reaction of ALS with NQS. Moreover, high pH may lead to hydrolysis of ALS, produced chromogen and NQS. Upon diluting the reaction solution with water, transparent solution was obtained indicating the solubility of ALS-NQS chromogen in water and the possibility of using water as a diluting solvent. In

order to select the most appropriate solvent for diluting the reaction solutions, different solvents were tested and compared with water; these solvents were methanol, ethanol, isopropanol, acetonitrile, acetone and methyl acetate. The highest absorbances were obtained when acetone or methyl acetate was used for dilution, but these absorbances were obtained without any distinguished peaks. However, the use of organic solvents leads to high analysis cost and more importantly, the incidence of exposure of the analysts to the side effects of these toxic solvents (*Fidler et al, 1987; Lindbohm et al, 1990; Wennborg et al, 2000; 2002 and Kristensen et al, 2008*). Moreover, in case of using inorganic bases in the reaction to make the alkaline media, best results were obtained when using NaOH and water as diluting solvent, where with other bases (sodium bicarbonate) either precipitation of white colloid, high blank readings, non reproducible results, and/or weak sensitivity were observed upon diluting the reaction solution with organic solvents, especially with isopropanol and ethanol. Therefore, water was used as diluting solvent in all subsequent experiments. The effect of temperature and time on the reaction was investigated by carrying out the reaction at room temperature (25 ± 3°C) and at elevated temperatures (40, 50 and 60 ± 3°C) for different periods of time (5, 10 and 15min). The results revealed that there was negligible difference between absorbances that have been obtained at room temperature and those at elevated temperatures up to 50°C, beyond which the absorbances decreased. Moreover, it was found that the reaction goes to almost completion within 5 min. In order to establish simple analytical procedures with no need for extra equipment (water bath) and for higher precision results; all subsequent experiments were carried out at room temperature for 10 min. Furthermore, the effect of reagents addition was investigated by carrying out the reaction in different orders. The results revealed that there was negligible difference between absorbances obtained in different orders of addition. The effect of time on the stability of ALS-NQS chromogen was also investigated by following the absorption intensity of the reaction solution after dilution at different time intervals up to 4 hours. It was found that the absorbance of the chromogen remains stable for at least 2 hours. This allowed the processing of large batches of samples with comfortable measurements. This gives the high throughput property to the developed method when applied for analysis of large number of samples in quality control laboratories. It has been reported that NQS could react with amino group of primary and secondary amine derivative (*Ali and Elbashir, 2012*) as well as amino group of primary and secondary amide derivative (*Babu et al, 2011 and Hadad et al, 2012*). Similarly, amino group of ALS at carbon atom no. 5, taking on nucleophilicity due to the lone

electron pair of nitrogen atom, trends to attack on the electron-deficient center in NQS, namely carbon atom no. 4 (3,4 carbon double bond can conjugate with carbon no. 2 and oxygen atom become electron rich, as a result carbon no.4 becomes electron deficient center). At the same time, it has been proved that the composition of ALS-NQS chromogen is 1:2 of ALS and NQS by limiting logarithmic method (*Rose, 1964*) and Job's method (*Job, 1964*). So it is concluded that terminal amine group of primary amide at carbon no. 2 in the side of nitrogen atom of octanamide in ALS reacted with other molecule of NQS. The primary amide was easily reacted than secondary amide due to steric hinders from methyl groups. According to the results obtained from limiting logarithmic and Job's methods that 2 molecules of NQS could react with primary amine and terminal amide group of ALS. The suggested reaction is shown in Figure 5.1.

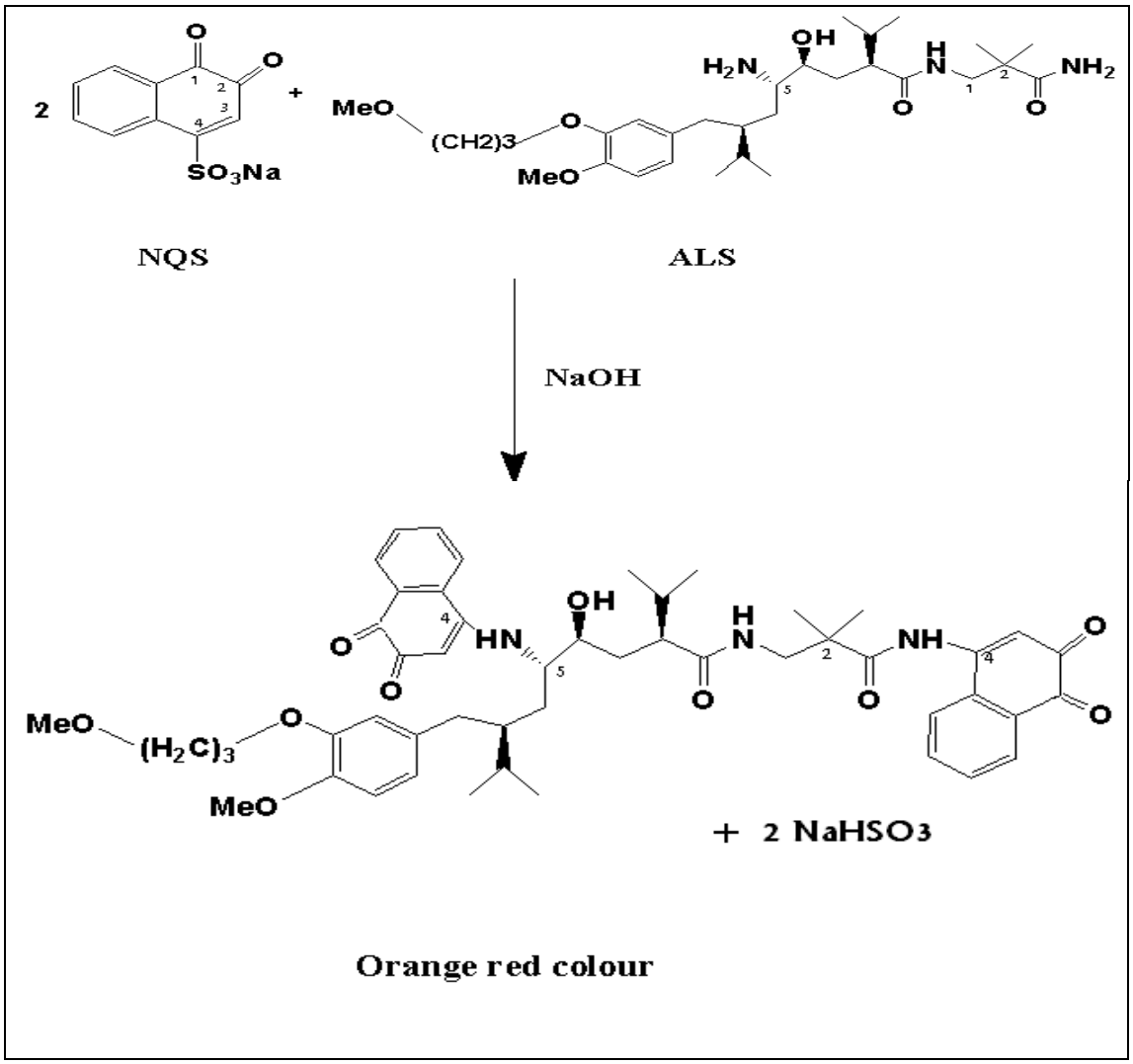

Figure 5.1: Suggested reaction of ALS with NQS.

Validation of the developed method was determined according to the ICH guidelines for validation of analytical procedures (*ICH, 2005*). Under the optimum reaction conditions, the calibration curve for the determination of ALS by its reaction with NQS was constructed. The regression equation for the results was A = 0.005 C + 0.1107 (R^2: 0.991), where A is the absorbance at 500 nm, C is the concentration of ALS in µg/ml and R^2 is the correlation coefficient. The linearity was observed in the concentration range of 20-300 µg/ml. The validity of the regression line was verified by statistical analysis, which demonstrated significant linear regression and non significant linearity deviation (p-value for slope and intercept < 0.05). The accuracy of the developed method was evaluated by the recovery studies for added concentrations. The recovery values were 99.17–100.65 ± 0.28–1.7% indicating the accuracy of the NQS method. Interferences liabilities were carried out to explore the effects of reagents used in the reaction and inactive ingredients that might be added during formulation. Comparison between ALS-NQS chromogen absorbance against blank and blank absorbance against water at 500 nm was carried out. The obtained figures showed that there was negligible absorbance of blank reagent at 500 nm, which indicated that there was no interferences of reagent blank at the same wavelength that the chromogen where measured. The interferences from inactive ingredients were investigated by mixing known amount (50 and 100 mg) of ALS with 50 or 10 mg of each common excipients alone and analyzing the samples by developed method procedures. The average recovery values for 50 and 100 mg of ALS were 99.95 ± 1.8% and 100.86 ± 1%, respectively. These data confirmed no interferences from any of the inactive ingredients in the determination of ALS by the developed method. The intra- and inter-assays of the developed method were also determined. The RSD did not exceed 0.62 and 1.7%, respectively; proving the high precision of this method for the routine application in the analysis of ALS in quality control laboratories. The influence of small variation in the method variables on its analytical performance was examined. It was found that small variation in the method variables did not significantly affect the procedures; recovery values were 98.2–102.78 ± 0.14–1.6%. This indicated the reliability of the method. Moreover, the inter-laboratory precision was tested and results obtained from lab-to-lab variations were reproducible, as RSD did not exceed 0.7%.

It is evident from the above-mentioned results that the developed method gave satisfactory results with ALS in bulk. Thus its pharmaceutical dosage form was subjected to the analysis of their ALS content by this method and reference method. Since there was neither official method nor reported derivatization spectrophotometric method for the

quantitative determination of ALS in its tablets, a validated direct spectrophotometric method was used as a reference method (*Wrasse-Sangoi et al, 2010*). The recovery percentage was 99.76 ± 0.57. This result was compared with that obtained from the reference method by statistical analysis. No significant difference was found at 95% confidence level proving similar accuracy in the determination of ALS by both methods.

5.2 NIN derivatization method

NIN is extensively used in the analytical determination of amino acids and potentially can react with a variety of primary and secondary amines producing *Ruhemann* purple color. In spectrophotometric determination, NIN had a wide range of application in determination of drugs. Despite of its old age of discovery, this method becomes a classical method. This method was based on the reaction of ALS with NIN at elevated temperature in slightly acidic conditions via oxidative deamination of the primary amino group followed by the condensation of the reduced NIN with other molecule of NIN to produce the purple colored chromogen which was measured at 569 nm.

Optimization of the reaction variables were investigated to achieve maximum color development and determined via a number of preliminary experiments. Studying the effect of NIN concentration and volume on its reaction with ALS revealed that the reaction was dependent on the NIN concentration as the absorbances increased with the increase in the reagent concentration. The highest absorbances were attained at a concentration range of 1.15-1.25% (w/v) (0.5ml) beyond which the absorbances decreased. Absorption decreased with high NIN concentration may be referred to reaction saturation, as well as decreased with higher volume due to dilution of the reaction. NIN is stable in acid at room temperature (often being recrystallised from HCL), but the stability decreased under basic conditions (*Schonberg and Azzam, 1939*). At high pH, the unprotonated amino group of the amino acids or the intermediate product can act as a good nucleophile, while at low pH these amino groups are protonated making them less nucleophilic, so that the reaction does not form the *Ruhemann* purple product in strong acidic conditions (*Joullie et al, 1991*). The optimum pH for the reaction of NIN with amino acids is around pH 5; however this varies with the amino acid (*Moore and Stein, 1948*). Two different types of buffer systems were investigated 0.2 M citrate and phosphate buffers at varying pH values (4.5-7). Best results were obtained in case of 0.2 M phosphate buffer pH 6.0. At higher pH values the absorbances decreased. Furthermore, the volume of this buffer system was also tested and found that, best results when 1 ml used for the reaction. It's believed that the first step in

the reaction is the oxidative deamination of amino group and the reduction of NIN then the reduced NIN condenses with other molecule of NIN to form purple colored product (*Lamothe and McCormick, 1973*). To prevent the oxidation of reduced NIN and to obtain consistent and reproducible results; the introduction of reducing agent was necessary. Different reducing agents were tested in this method. These agents were ascorbic acid, sodium molybdate and zinc chloride prepared in concentration of 0.1, 0.2 and 1%. Best results were obtained in the case of ascorbic acid in 0.1% (1ml). However, the obtained results with ascorbic acid were lower than those without it, so reducing agents was excluded in subsequent experiments. Transparent solution was obtained upon diluting the reaction solution with water, indicating the solubility of ALS-NIN chromogen in water and the possibility of using water as a diluting solvent. In order to select the most appropriate solvent for diluting the reaction solutions, different solvents were tested and compared with water; these solvents were methanol, ethanol, isopropanol, acetonitrile and ethyl acetate. The highest absorbance was obtained when water was used for dilution. Therefore, we can avoid organic solvents and water was used as diluting solvent in all subsequent experiments. ALS reacted with NIN only at higher temperature. Color was developed from the reaction at room temperature after 4 hours. The effect of temperature and time on the reaction was investigated by carrying out the reaction at (80, 90 and 100 ± 3°C) for different periods of time (5, 10, 15, 20 and 25min). Best results were obtained at 90°C for 20 min, which emphases the importance of heating on the reaction. To avoid the effect of reagents addition, 1.2% NIN in 0.2 M phosphate buffer pH 6.0 was prepared. This solution was used for construction of calibration curve. The obtained calibration curve was with wide range of beer's law limits, but without higher sensitivity from other methods and good reproducible results. Several combination solutions were investigated to increase the sensitivity and to obtain higher reproducible measurements. 1 ml of ascorbic acid solution 0.1% was added to 1 ml of previous solution and constructed a calibration curve. The beer's law limits were decreased without increase in the sensitivity, but had more reproducible measurements. Other combination solution was prepared from 1.2% NIN mixed with 0.1% ascorbic acid dissolved in 0.2 M phosphate buffer pH 6.0 (N/A/PH). The beer's law limits were decreased with higher increase in the sensitivity and reproducibility. Furthermore, two solutions were tested; 1 ml 1.2% NIN with 0.1% ascorbic acid dissolved in water mixed with 0.5 ml 0.2 M phosphate buffer pH 6.0 and 1 ml 1.2% NIN with 0.03% ascorbic acid dissolved in 0.2 M phosphate buffer pH 6.0. The two solutions had higher reproducibility, but the former didn't increase the sensitivity and the other increased the

sensitivity but beer's law limits was sharply decreased. From previously mentioned results, the combination of 1.2% NIN mixed with 0.1% ascorbic acid dissolved in 0.2 M phosphate buffer pH 6.0 was used, which have increase in sensitivity with good range of beer's law limits and higher reproducible measurements. Moreover, the effect of volume of this solution was investigated. The results revealed that the absorbances increased with the increase of volume. However, the maximum absorbance of product chromogen was 1.53 A°, which was by using 2 ml of this solution for 100 µg/ml of ALS. To increase the beer's law limits, 1 ml of this solution was used for subsequent experiments. The effect of time on the stability of ALS-NIN chromogen was also investigated by following the absorption intensity of the reaction solution after dilution at different time intervals up to 4 hours. It was found that the absorbance of the chromogen remains stable for at least 1.5 hours. This allowed the processing of large batches of samples with comfortable measurements. This gives the high throughput property to the developed method when applied for analysis of large number of samples in quality control laboratories. Under the optimum reaction conditions, the stoichiometry of the reaction between ALS and NIN was investigated by limiting logarithmic method (*Rose, 1964*) and Job's method (*Job, 1964*). The obtained results confirm that the ratio between ALS and NIN was 1:2. The reaction proceeds via oxidative deamination of the primary amino group of ALS at carbon no. 5 followed by the condensation of the reduced NIN with other molecule of NIN. The suggested reaction is shown in Figure 5.2.

Validation of the developed method was determined according to the ICH guidelines for validation of analytical procedures (*ICH, 2005*). Under the optimum reaction conditions, the calibration curve for the determination of ALS by its reaction with NIN was constructed. The regression equation for the results was $A = 0.008\ C + 0.161$ (R^2: 0.992), where A is the absorbance at 569 nm, C is the concentration of ALS in µg/ml and R^2 is the correlation coefficient. The linearity was observed in the concentration range of 10-170 µg/ml. The validity of the regression line was verified by statistical analysis, which demonstrated significant linear regression and non significant linearity deviation (p-value for slope and intercept < 0.05). The accuracy of the developed method was evaluated by the recovery studies for added concentrations. The recovery values were 99.63–101.2 ± 0.25–1.27% indicating the accuracy of the NIN method. Interferences liabilities were carried out to explore the effects of reagents used in the reaction and inactive ingredients that might be added during formulation. Comparison between ALS-NIN chromogen absorbance against blank and blank absorbance against water at 569 nm was carried out.

The obtained figures showed that the blank reagent didn't have absorbance at 569 nm, which indicated that there was no interferences of reagent blank at the same wavelength that the chromogen where measured.

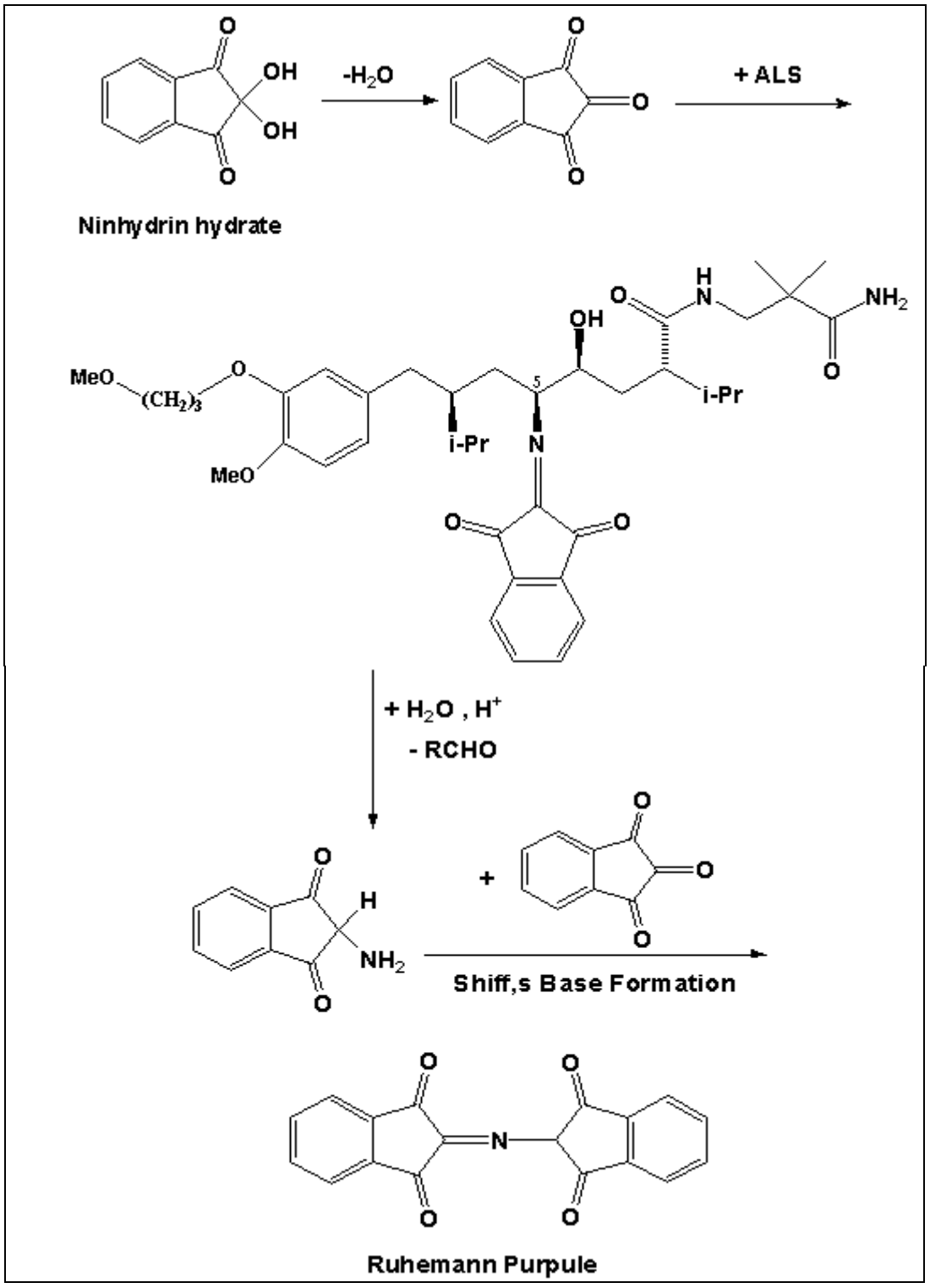

Figure 5.2: Suggested reaction of ALS with NIN.

The interferences from inactive ingredients were investigated by mixing known amount (50 and 100 mg) of ALS with 50 or 10 mg of each common excipients alone and analyzing the samples by developed method procedures. The average recovery values for 50 and 100 mg of ALS were 99.13 ± 0.76% and 100.3 ± 0.82%, respectively. These data confirmed the absence of interferences from any of the inactive ingredients in the determination of ALS by the developed method. The intra- and inter-assays of the developed method were also determined. The RSD did not exceed 1.6% and 1.46%, respectively; proving the precision of this method for the routine application in the analysis of ALS in quality control laboratories. The influence of small variation in the method variables on its analytical performance was examined. It was found that small variation in the method variables did not significantly affect the procedures; recovery values were 98.6–101.1 ± 0.31–1.12%. This indicated the reliability of the method. Moreover, the inter-laboratory precision was tested and results obtained from lab-to-lab variations were reproducible, as RSD did not exceed 1.85%.

It is evident from the above-mentioned results that the developed method gave satisfactory results with ALS in bulk. Thus its pharmaceutical dosage form was subjected to the analysis of their ALS content by this method and reference method (*Wrasse-Sangoi et al, 2010*). The recovery percentage was 100.15 ± 1.29. This result was compared with that obtained from the reference method by statistical analysis. No significant difference was found at 95% confidence level proving similar accuracy in the determination of ALS by both methods.

The comparison between NQS, NIN and reference method (*Wrasse-Sangoi et al, 2010*) based on the analysis of variance (One way ANOVA test) demonstrated that, their were no significant difference between them and providing similar precision in the determination of ALS content in pharmaceutical dosage form.

Chapter 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

- The present study described, for the first time, the successful evaluation of NQS and NIN as derivatization reagents in the development of simple, sensitive and inexpensive spectrophotometric methods for the accurate determination of ALS in pharmaceutical dosage forms.
- The NQS method was based on the reaction of ALS with NQS in alkaline medium producing an orange-red colored product which absorbs maximally at 500 nm, while NIN method was based on the reaction of ALS with NIN mixed with ascorbic acid as reducing agent in phosphate buffer pH 6.0 producing blue-violet colored product which absorbs maximally at 569 nm.
- The experimental parameters for methods were studied and optimized. The optimum conditions were 1 ml 0.5% NQS solution, 1 ml 0.01 M NaOH solution as alkaline media, water as diluting solvent and 10 min reaction time maintained at room temperature for NQS method and 1 ml 1.2% NIN mixed with 0.1% ascorbic acid dissolved in 0.2 M phosphate buffer pH 6.0, water as diluting solvent and 20 min reaction time maintained at 90±3°C for NIN method.
- The products chromogen of NQS and NIN methods remained stable for at least 2 and 1.5 hours, respectively. This gives the high throughput property to the developed methods when applied for analysis of large number of samples in quality control laboratories.
- The stoichiometry of the reactions between ALS: NQS and ALS: NIN was in both cases 1:2, according to limiting logarithmic (*Rose, 1964*) and Job's methods (*Job, 1964*).
- The regression equations for NQS and NIN methods were $A = 0.005\ C + 0.1107$ (R^2: 0.991) and $A = 0.008\ C + 0.161$ (R^2: 0.992), respectively.
- The molar absorptivity for NQS and NIN methods were 3.25×10^5 and 5.3×10^5, respectively.
- The developed methods were validated according to ICH guidelines (*ICH, 2005*), for linearity, range, accuracy, specificity, precision, LOD, LOQ, robustness and ruggedness.

- The linear ranges of NQS and NIN methods were 20-300 and 10-170 μg/ml, respectively, which are more sensitive than some reported methods 40-100 μg/ml (*Wrasse-Sangoi et al, 2010*) and 60-1200 μg/ml (*Sangoi et al, 2011*).
- The linear range of NIN method (10-170 μg/ml) is comparable with other methods 10-300 μg/ml (*Wrasse-Sangoi et al, 2011*) and 10-50 μg/ml (*Swamy et al, 2012[a]*) with advantages of simple, inexpensive and rapid analysis technique in comparison with highly cost and sophisticated instruments. Moreover, less environment toxic method, employing mainly distilled water, as solvent.
- Regarding accuracy, the recovery values for NQS and NIN methods were 99.17–100.65 ± 0.28–1.7% and 99.63–101.2 ± 0.25–1.27%, respectively.
- Interferences liabilities from reagents used and inactive ingredients "excipients" were absence in both developed analytical procedures.
- The RSD for intra- and inter-assay precisions for NQS and NIN methods did not exceed 0.62 and 1.7% as well as 1.6% and 1.46%, respectively.
- The LOD and LOQ for NQS and NIN methods were 1.6 and 4.85 as well as 1.27 and 3.85 μg/ml, respectively.
- The influence of small variation in the methods variables did not significantly affect the procedures; recovery values for NQS and NIN methods were 98.2–102.78 ± 0.14–1.6% and 98.6–101.1 ± 0.31–1.12%, respectively.
- Results obtained from lab-to-lab variations were reproducible, as RSD for NQS and NIN methods did not exceed 0.7% and 1.85%, respectively.
- The pharmaceutical dosage form of ALS was subjected to the analysis of their ALS content by developed methods and a reference one (*Wrasse-Sangoi et al, 2010*). The results were compared by statistical analysis with respect to the accuracy and precision and no significant differences were found.

6.2 Recommendations

- The new analytical methods depend greatly on highly qualified instruments, which make the cost very high. The UV methods are inexpensive, fast and produce very low levels of dangerous residues promoting benefits to the public health and the environment.
- Application of derivatization reaction in drug analysis, especially when weak or no chromophore is present.

- Spectrophotometric determination of a drug after derivatization reaction achieves a comparable sensitivity limits with the highly expensive and sophisticated instrumental analysis.
- Development of such spectrophotometric analysis should avoid the high cost of recently published analytical procedure, which is an advantage to the developing countries.
- The developed methods are practical and simple and can be applied for aliskiren determination in pharmaceutical dosage forms in quality control laboratories.
- Further studies can be performed using the described procedures for stability study of aliskiren.
- The applicability of new procedures for routine quality control of aliskiren in bulk and pharmaceutical formulations.

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