Sudan University of Science and Technology
College Of Graduate Studies

Design and validation of chromatographic and spectrophotometric stability indicating methods for determination of amlodipine besylate and esomeprazole magnesium

تصميم و تحقق من طرق كروماتوغرافية و مضوائية طيفية دالة علي الثباتية لتعيين الأملودبين بيزيلات و الإيزوميبرازول مغنسيوم

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Dedication

I dedicate this work to my parents, and friends;

without them none of my success would be possible

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Abstract

The objectives of this study were both to develop, optimize and validate spectrophotometric and chromatographic methods for the analysis of amlodipine besylate and esomeprazole magnesium in pharmaceutical formulations in presence of their degradation products and to study their stability under various conditions.

The spectrophotometric method for amlodipine was based on either its solubility enhancement using different hydrotropic agents including potassium acetate, ammonium acetate and nicotineamide, or on its formation of ion-pair complex with methyl orange at pH 3.5, showing percentage recovery ranges of 99.74 – 100.04 and 99.82 – 101.05, respectively. For esomeprazole magnesium, the method, however, was based either on its reaction with alizarin red s to form a deep brown complex, or on its formation of ion-pair complex with eriochrome black T at pH 3, showing percentage recoveries ranges of 99.53 – 100.45 and 99.57- 100.52, respectively. These recovery results indicated that both developed spectrophotometric methods for each drug were highly accurate.

The development of the liquid chromatographic methods for each drug, however, was based on the application of the factorial design approach, and they were all performed at ambient temperature; for amlodipine besylate, the mobile phase used was composed of either of acetonitrile and water (60:40 v/v) in presence of 0.1% triehylamine or of 20 mM potassium dihydrogen phosphate, flowing at a rate of either 1.0 or 1.5 ml/min, through column C_{18} column (containing 5µm- size particle) of dimensions 250× 4.5 mm or 150× 4.6 mm, respectively at wavelength either 362 or 212 nm, giving recovery percentage of either 99.96 – 100.54 or 99.76 – 101.13, respectively. For esomeprazole magnesium, the mobile phase used was composed of water and either methanol (60:40) or ethanol (45:55), flowing at the same flow rate of 1.0 ml/min for both methods, through either column C_{12} (150× 4.5 mm) or column C_{18} column (150× 4.6 mm), both containing 5µm-size particles, respectively at 302 nm wavelength for both methods, and giving percentage recovery range of 99.90 – 100.26 and 99.43 – 100.78, respectively. These recovery results for both developed chromatographic methods for each drug were also highly accurate.

It was found that stability of both drugs was affected by several factors including hydrolysis, heat, and sunlight. The kinetic study of the effect of sunlight, thermal hydrolysis, hydrolysis in alkaline medium and effect of heat on the stability of amlodipine besylate revealed that the reaction was zero, second, zero and first order, respectively. That of the effect of sunlight, thermal hydrolysis, hydrolysis in acidic medium and heat on the stability of esomeprazole magnesium revealed that the reaction was zero, zero, first and zero order, respectively.

الملخص

الهدف من هذه الدراسه هو تطوير و تحسين طرق مضوائية طيفية و كروماتوغرافيه لتحليل كل من الأملودبين بيزايلات و الإيزوميبرازول مغنسيوم في المستحضرات الصيدلانيه في وجود نواتج تفككهما عند الظروف المختلفه.

تعتمد الطرق المضوائية إما علي: زيادة ذوبانية الأملودبين بإستخدام عوامل هيدروتروبية مختلفة تشمل خلات البوتاسيوم, خلات الأمونيوم و النيكوتين أميد أو علي تكوين معقد ثنائي الأيون بين الأملودبين و الميثيل البرتقالي عند الرقم الهيدروجيني 3.5. وجد أن نسبة الإسترداد تتراوح في المدي 99.47 – 100.04 و 99.82 – 101.05 لكل من الطريقتين علي التوالي. بالنسبة للإيزوميبرازول مغنسيوم تعتمد إما علي: التفاعل بين الإيزوميبرازول و الأليزارين الأحمر لتكوين معقد بني غامق أو علي تكوين معقد ثنائي الأيون بين الإيزوميبرازول و الإيروكروم بلاك تي عند الرقم الهيدروجيني 3. وجد أن نسبة الإسترداد تتراوح في المدي 99.53 – 100.45 و – 100.52 99.57 للطريقتين علي التوالي. تؤكد نتائج نسبة الإسترداد علي صحة الطريقتين.

تم تطوير طرق كروماتوغرافية لكلا العقارين بتطبيق التصميم العاملي. بالنسبة الأملودبين بيزايلات تم إستخدام طور متحرك يتكون من الأسيتونتريل و الماء (40:60) في وجود 0.1% ثلاثي إيثيل أمين أو 20 مليمولار فوسفات البوتاسيوم ثنائي الهيدروجين بمعدل سريان 1.0 و 1.5 مل/دقيقة خلال عمود فصل C_{18} (5 ميكرومتر) بابعاد 250×4.6 ملم أو 150×4.6 ملم علي التوالي عند طول موجي 362 و 212 نانومتر علي التوالي فكانت نسبة الإسترداد في المدي 99.46 – 100.54 و 99.76 – 101.13علي التوالي. بالنسبة للإيزوميبرازول مغنسيوم يتكون الطور المتحرك من الماء و إما الميثانول (60:40) أو الإيثانول (55:45) بمعدل سريان 1.0 مل/دقيقة لكل من الطريقتين عبر عمود C_{12} (150×4.5 ملم, 5 ميكرومتر) أو C_{18} (150×4.6 ملم, 5 ميكرومتر) لكل من الطريقتين علي التوالي عند طول موجي 362 نانومترلكل من الطريقتين, لتعطي نسبة إسترداد في المدي 100.26 99.9 – و 99.43 – 100.78 علي التوالي. تؤكد نسبة الإسترداد علي صحة الطريقتين.

وجد أن ثباتية كل من العقارين تتأثر بعدة عوامل تشمل التحلل الحراري, الحراره و ضوء الشمس . دراسة حركية تأثير كل من ضوء الشمس, التحلل الحراري, التحلل الحراري في الوسط القاعدي و الحراره علي ثباتية الأملودبين بيزايلات كشفت أنها من الرتب الصفريه, الثانيه, الصفريه و الأولي علي التوالي. تأثير ضوء الشمس و التحلل الحراري, التحلل الحراري في وسط حمضي و الحراره علي ثباتيه الايزوميبرازول كشفت أنها من الرتب الصفريه, الصفريه , الثانيه والصفريه علي التوالي.

List of publications

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Table of Contents

Chapter two

Chapter Three

List of tables

List of figures

List of Abbreviations

API	Active pharmaceutical ingredient
B.P	British Pharmacopeia
FDA	Food and Drug Administration
FID	Flame ionization detector
HPLC	High Performance Liquid Chromatography
HPTLC	High performance thin layer chromatograph
ICCBS	International center for chemical and biological science
ICH	International Conference of Harmonization
IR	Infrared
LOD	Limit of detection
LOQ	Limit of quantitation
M.O	Methyl orange
NLT	Not less than
NMT	Not more than
RI	Refractive index
RP	Reverse phase
RSD	Relative standard deviation
SD	Standard deviation
TEA	Triehylamine
TWAS	Third world academy of sciences
USP	United states Pharmacopeia
UV	Ultraviolet
VIS	Visible light

1 INTRODUCTION

1.1 Method development

Analytical method development aims to improve chemical analysis techniques and is commonly associated with method assessment and validation in industry and government agencies. Method development requires application of skills involving critical thinking and problem solving (Lanigan, 2008).

A review on method development indicates that individual approaches exist which are based on previous experience of compounds or previous knowledge; method development often follows the same sequence of events which are summarized as:

1.1.1 Definition of the problem

Before the analyst can design an analytical procedure, one must know what information is needed, by whom, for what purpose, and what type of sample is to be analyzed. Once the problem is defined this will dictate how the sample is to be obtained, how much is needed, how sensitive the method must be, how accurate and precise it must be, and what separations may be required to eliminate interferences (Valcarcel, 2000; Christian, 2004).

1.1.2 Sampling

A chemical analysis is usually performed on only a small portion of the material to be characterized. It may be homogeneous or heterogeneous in composition. In the former case, a simple “grab sample” taken at random will suffice for the analysis. In the latter, we may be interested in the variation throughout the sample; in such case several individual samples will be required.

Certain precautions should be taken in handling and storing samples to prevent or minimize contamination, loss, decomposition, or matrix change. In general one must prevent contamination or alteration of the sample by the container, the atmosphere, or light. Also the stability of the sample must be considered (Kealey and Haines, 2002).

1.1.3 Preparation of sample for analysis

The first step in analyzing a sample is to measure the amount being analyzed. The analytical sample size must be measured to the degree of precision and accuracy required for the analysis. The amount of sample taken will depend on the concentration of the analyte and how much is needed for isolation and measurement. Determination of major constituent may require only a couple of hundred milligrams of sample, while trace constituent may require several grams. Usually replicate samples are taken for

analysis in order to obtain statistical data on the precision of the analysis to provide more reliable results.

More often the sample must be in solution form for measurement; once a sample is in solution, the solutions conditions must be adjusted for the next stage of the analysis. The analyte may have to be reacted with a reagent to convert it to a form suitable for measurement or separation.

The solvents and reagents used for dissolution and preparation of the solution should be of high purity. Even so they may contain trace impurities of the analyte. Hence it is important to prepare and analyze replicate blanks, particularly for trace analysis. The blank result is subtracted from the analytical sample result to arrive at a net analyte concentration in the sample solution. If the blank is appreciable, it may invalidate the analysis(Kealey and Haines, 2002; Skoog *et al.*, 2014).

1.1.4 Separation of interferences

In order to eliminate interferences, to provide suitable selectivity in the measurement, or to preconcentrate the analyte for more sensitive or accurate measurement, the analyst must often perform one or more separation steps. It is preferable to separate the analyte away from the sample matrix, in order to minimize losses of the analyte. Separation steps may include precipitation, extraction into an immiscible solvents, chromatography, and distillation (Fifield and Kealey, 2000).

1.1.5 Measurement

The method employed for actual quantitative measurement of the analyte will depend on a number of factors, including the amount of analyte present and the accuracy and precision required.

Many available techniques possess varying degrees of selectivity, sensitivity, accuracy and precision, cost and rapidity. The techniques available are gravimetric analysis, volumetric analysis, and instrumental analysis. Instrumental techniques are generally more sensitive and selective than the classical techniques but are less precise, they are usually more rapid, may be automated, and may be capable of measuring more than one analyte at a time (Christian, 2004).

The selection of a technique, when more than one is applicable, will depend on the availability of equipment and personal experience and preference of the analyst (Christian, 2004; Skoog *et al*., 2014).

1.1.6 Calculation and data reporting

Once the concentration of analyte in the prepared sample solution has been determined, the results are used to calculate the amount of analyte in the original sample. Either an absolute or a relative amount may be reported. Replicate analysis can be performed, and precision of the analysis may be reported. Knowledge of precision is important because it gives the degree of uncertainty in the result. The analyst should critically evaluate whether the results are reasonable and related to the analytical problem as originally stated (Christian, 2004).

1.2 Spectrophotometric method development

Several ultra violet/visible Spectrophotometric tests have been widely used in chemical analysis. The common availability of the instrumentation, the simplicity of procedures, economy, speed, precision and accuracy of the technique still make spectrophotometric methods attractive (Bonfilio *et al.*, 2010; Rojas and Ojeda, 2004).

1.2.1 Instrumentation

A spectrophotometer is an instrument which is capable of isolating **'**monochromatic**'** radiation, or that which specifically contains a dispersing element: a prism or a grating. It is pertinent to mention here that there are a plethora of commercially available spectrophotometers of varying design. Single-beam (simple), double-beam (more precise and accurate) and microcomputer controlled built-in-recorder with separate printer. In single-beam spectrophotometer the desired wavelength is isolated by using a prism or grating and auxiliary mirrors and slits that collectively form a microchromator of the instrument. The wavelength dial on a spectrophotometer is adjusted to a specific value, but the radiation leaving the exit-slit is found to be rarely monochromatic.

The quantum leap amalgamated with qualified success in the advancement of analytical instruments necessitated for more rapid, precise and accurate measurements in UV and visible spectroscopy. It could be accomplished by the help of the following two cardinal modifications, namely:

- Need for a continuous change in wavelength so that light through the blank and through the sample may be monitored continuously.
- Measurements done with a recording spectrophotometer.

The above two modifications have been dually incorporated in a double-beam spectrophotometer (Kar, 2005).

1.2.2 Assay methods

UV-Visible spectroscopic data can give qualitative and quantitative information of a given compound or molecule. Irrespective of whether quantitative or qualitative information is required it is important to use a reference cell filled with the blank solution to zero the instrument. For quantitative information of the compound, calibrating the instrument using known concentrations of the compound in question in a solution with the same solvent as the unknown sample would be required. If the information needed is just to prove that a compound is in the sample being analyzed, a calibration curve will not be necessary; however, if a degradation study or reaction is being performed, and concentration of the compound in solution is required, thus a calibration curve is needed. To make a calibration curve, at least three concentrations of the compound will be needed, but five concentrations would be most ideal for a more accurate curve. The concentrations should start at just above the estimated concentration of the unknown sample and should go down to about an order of magnitude lower than the highest concentration. The calibration solutions should be spaced relatively equally apart, and they should be made as accurately as possible (Gandhimathi *et al.*, 2012; ICH, 2005).

1.2.3 Sample preparation

The substance under examination is usually dissolved in a spectroscopic grade solvent. Particular care must be taken to employ solvents free from contaminants absorbing in the specific spectral region being used. The ideal solvent for the preparation of sample solutions would dissolve all types of compounds, would be nonflammable and nontoxic, and would be completely transparent at all wavelengths. The solvent chosen for this dissolution process may be either polar or nonpolar depending on the polarity and reactivity of the sample. Every solvent has a UV-visible absorbance cutoff wavelength. The solvent cutoff is the wavelength below which the solvent itself absorbs all of the light. So when choosing a solvent be aware of its absorbance cutoff and where the compound under investigation is thought to absorb. If they are close, chose a different solvent (Cullum and Sicker, 2010; Gandhimathi *et al.*, 2012).

In some cases chemical derivatiztion is used to improve sensitivity or selectivity. Also for poorly soluble compounds in water a number of methodologies can be adapted to improve solubilization of poor water soluble drug and further to improve its bioavailability. The techniques generally employed for solubilization of drug includes

micronization, chemical modification, pH adjustment, solid dispersion, complexation, co-solvency, micellar solubilization, and hydrotropy.

1.2.3.1 Chemical derivatiztion methodologies

Chemical derivatiztion is done by conversion of functional groups within the molecule to other more readily adaptable groups to the technique of analysis. This technique is applied in UV-VIS spectroscopy, gas chromatography and high performance liquid chromatography.

Wide ranges of reaction have been adopted for chemical derivatiztion. Majority of these reactions are color producing reactions. Some of these reactions have wide applications in chemical derivatiztion for UV-VIS spectrophotometric determination of pharmaceuticals. The major reactions are: azo dye formation, shiff-base formation, charge transfer complexation, ion-pair formation, complexation reactions, oxidation – reduction reactions, and miscellaneous methods (Adegoke, 2012).

1.2.3.1.1 Ion-pair formation

Majority of ion-pair formations are carried out between drugs and dyes molecules at pH values where dyes can serve as charge donors. Subsequently, the ion-pairs are extracted into organic solvents and spectra overlay is carried to determine the new wavelength of maximum absorption. Chloroform, being highly nonpolar and highly immiscible with water has found the greatest relevance in extraction processes of ion-pair complexes. Bromocresol blue, Bromocresol purple, and Bromocresol green have found the greatest application as ion-pair donors in most reported methods. Other ion-pair donors used are methyl orange, bromophenol dyes, and alizarin red s (Wahbi *et al.*, 1993).

1.2.3.1.2 Miscellaneous methods

Other reagents have been used for determination of wide range of pharmaceuticals are 4-Chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl), Sodium 1,2-naphthoquinone-4-sulphonic acid (NQS), alizarin red s, which are used for pharmaceutical amines (both primary and secondary amines) , sodium nitroprusside (SNP) which is used for thiols and sulphur containing compounds. In most cases condensation reaction occurs (Wahbi *et al.*, 1993; Haggag *et al.*, 2008).

1.2.3.2 Hydrotropy

Hydrotropy is a solubilization process whereby addition of a large amount of second solute results in an increase in the aqueous solubility of another solute. Additives or salts that increase solubility in given solvent are said to “salt in” the solute and those salts that decrease solubility “salt out” the solute. Several salts with large anions or cations that

are themselves very soluble in water result in “salting in” of non electrolytes called “hydrotropic salts”: a phenomenon known as “hydrotropism”. Hydrotropic solutions do not show colloidal properties and involve a weak interaction between the hydrotropic agent and solute. The mechanism by which it improves solubility is more closely related to complexation involving a weak interaction between the hydrotropic agents like sodium benzoate, sodium salicylate, sodium acetate, sodium alginate, urea, nicotinamide, sodium citrate and the poorly soluble drugs (Vemula *et al.,* 2010).

Typically, hydrotropes consist of a hydrophilic part and a hydrophobic part (like surfactants) but the hydrophobic part is generally too small to cause spontaneous self-aggregation. Hydrotropes do not have a critical concentration above which self aggregation 'suddenly' starts to occur. On the other hand, planarity of the hydrophobic part has been emphasized as an important factor in the mechanism of hydrotropic solubilization. This should imply that hydrotropic agents are molecules having a planar hydrophobic structure brought into solution by a polar group. Hence, it seems rational to propose that molecules with a planar hydrophobic part and a polar group, which is not necessarily anionic, can act as hydrotropic agents. It was suggested that the phenomenon of hydrotropy is not confined to the metal salts of organic acids, certain cationic salts and neutral molecules may be equally involved (Jain *et al.*, 2010; Nidhi *et al.*, 2011).

The classification of hydrotropes on the basis of molecular structure is difficult, since a wide variety of compounds have been reported to exhibit hydrotropic behaviour. Specific examples may include aromatic alcohols like resorcinol, pyrogallol, catechol, and β-naphthols and salicylates, alkaloids like caffeine and nicotine, ionic surfactants like diacids,(sodium dodecyl sulphate) and dodecylated oxidibenzene. The aromatic hydrotropes with anionic head groups are mostly studied compounds. They are large in number because of isomerism and their effective hydrotrope action may be due to the availability of interactive pi-orbitals. Hydrotropes with cationic hydrophilic group are rare, like salts of aromatic amines, such as procaine hydrochloride (Vemula *et al.*, 2010).

The advantages of hydrotropic solubilization technique are:

- Hydrotropy is suggested to be superior to other solubilization method, because the solvent character is independent of pH, has high selectivity and does not require emulsification.
- It only requires mixing the drug with the hydrotropes in water.

- It does not require chemical modification of hydrophobic drugs, use of organic solvents, or preparation of emulsion system.

1.3 HPLC method development

High performance liquid chromatography (HPLC) was derived from the classical column chromatography and, is one of the most important tools of analytical chemistry today. The principle is that a solution of the sample is injected into a column of a porous material (stationary phase) and a liquid (mobile phase) is pumped at high pressure through the column. The separation of sample is based on the differences in the rates of migration through the column arising from different partitions of the sample between the stationary and mobile phase. Depending upon the partition behavior of different components, elution at different times takes place. High performance liquid chromatography is more versatile than gas chromatography since it is not limited to volatile and thermally stable samples, and the choice of mobile and stationary phases is wider (Gupta, *et al.*, 2012).

A review of HPLC method development indicates that individual approaches exist which are based on previous experience of compounds or previous knowledge; method development often follows the same sequence of events. These are summarized in the following steps.

1.3.1 Information on the sample

If some information on the nature of the sample is known before beginning method development, it often provides valuable clues to the choice of separation technique, detection mode, extraction procedure (if required) and initial starting conditions for the separation (Okafo and Robert, 2003).

For method development one has to study the physical properties like solubility, polarity, spectral data, pka and pH, partition coefficients, fluorescent properties (if any), chromatographic behavior, spectrophotometric properties, and oxidation-reduction potential of the sample molecule.

pH and pka play an important role in HPLC method development. Based on pH or pKa values the nature of the compound and polarity of the compound can be assumed. When pH is equivalent to pKa, the compound is half ionized. Almost all the pH related change occurs within ± 1.5 units of the pKa value. Outside this range the compound is either ionized or non-ionized, and its retention does not change much with pH. The ionogenic nature of the compound of interest should be determined. If the target analyte

is neutral, the eluent pH will not affect its retention. However, the structure of this neutral molecule must be assessed, to postulate if a potential ionogenic degradation product may be formed during stress testing and stability testing. If this is the case, the HPLC method must be capable of adequately retaining and separating this "potential ionogenic" species from the active and other degradation products or impurities (Phani *et al.*, 2012).

The solubility of molecules can be explained on the basis of the polarity of molecules. Selection of diluents is based on the solubility of analyte. The analyte must be soluble in the diluents and must not react with any of the diluent components. The diluent should match to the starting eluent composition of the assay to ensure that no peak distortion will occur, especially for early eluting components. If the analyte is more soluble in the diluent than the starting eluent composition, the compound will tend to reside in the "solvent plug" being injected onto the column and a peak fronting or skewing may occur. The data on fluorescence, spectrophotometric, chromatographic, and oxidation-reduction properties can be used to determine the best means of measuring and quantifying the analyte of interest (Gupta, *et al.*, 2012; Kaushal and Srivastava, 2010).

1.3.2 Sample pretreatment

Sample preparation is an essential part of the separation process and is one of the key considerations at the start of any method development strategy. The primary aim is to provide a reproducible and homogenous solution that is ready for injection into the separation system. Samples can be presented in various forms as solids, liquids, suspensions and even gases. In the solution form, these samples can either be ready for direct injection into the separation system or require further manipulation, like dilution, buffering or addition of internal standards. When samples are in the solid form, they must first be either dissolved or extracted using a suitable solvent. In circumstances where the sample is relatively soluble, direct dissolution in a compatible solvent is the preferred mode of sample preparation because it is convenient and offers greater precision. For these sample types, the choice of solvent is important because choosing a solvent system that closely matches the separation solvent minimizes baseline problems and other unwanted separation effects (Okafo and Robert, 2003).

1.3.4 Detector selection and setting

A detector is an important part of the liquid chromatography, it should be chosen very carefully for selective separation and accurate determination. The single most crucial factor is continuous detection based on the progress of separation of a component which

may be immediately displayed and then recorded. However, a good detector should have linear response to solutes that extends over several orders of magnitude. It must have good stability, reproducibility and reliability. It should have low dead volume to minimize extra-column band broadening (Phani *et al.*, 2012).

Selection of detector depends on the chemical nature of analyte, potential interference, limit of detection required, availability and/or cost of detector. Different types of detectors are available such as ultraviolet (UV), fluorescence, electrochemical, light scattering, refractive index (RI), flame ionization detection (FID), evaporative light scattering detection (ELSD), corona aerosol detection (CAD), mass spectrometric (MS), NMR, and others. However, the majority of reversed-phase and normal phase HPLC method development in the pharmaceutical industry is carried out with either refractive index or UV-visible detectors (Gupta, Jain, *et al.*, 2012; Kaushal and Srivastava, 2010).

1.3.5 Choice of stationary phase (column)

The heart of a HPLC system is the column. Changing a column will have the greatest effect on the resolution of analytes during method development. In general, the nature of stationary phase has the greatest effect on capacity factor, selectivity, efficiency and elution. There are several types of matrices for support of the stationary phase, including silica, polymers, and alumina. Silica is the most common matrix for HPLC columns. Silica matrices are robust, easily derivatized, manufactured to consistent sphere size, and does not tend to compress under pressure. Silica is chemically stable to most organic solvents and to low pH systems. One shortcoming of a silica solid support is that it will dissolve above pH 7. In recent years, silica supported columns have been developed for use at high pH (Gupta, *et al.*, 2012).

In reverse phase chromatography (RP-HPLC) the stationary phase is non-polar and the mobile phase is polar, causing polar peaks to generally elute earlier than non-polar peaks. To create a stationary phase for RP-HPLC on silica support, the free silanols are reacted with a chlorosilane with hydrophobic functionality to introduce the non-polar surface. Due to steric constraints, only about 1/3 of the surface silanols are derivatized. The remaining free silanols can interact with analytes, causing peak tailing. Typically, after the derivitization of a column with the desired stationary phase, the column is further reacted with chlorotrimethylsilane to end cap the remaining free silanols and improve the column efficiency. Common stationary phases are C4 (butyl), C8 (octyl), C18 (octadecyl), nitrile (cyanopropyl), and phenyl (phenyl propyl) columns. However,

in normal phase analysis the column is more polar when compared to mobile phase: cyano, phenyl, silica and chiral columns (Phani *et al.*, 2012).

The criteria for selection may include that the column is stable for a certain number of column volumes at the recommended maximum and minimum pH at a particular maximum temperature. Also column parameters, like column dimension, particle shape, particle size, surface area, pore size, bonding type, carbon load, and endcapping, are to be checked to verify system suitability criteria (Kaushal and Srivastava, 2010).

1.3.6 Choice of mobile phase

Mobile phase composition (or solvent strength) plays an important role in RP-HPLC separation. It affects resolution, selectivity and efficiency. In RP-HPLC, the mobile phase consists of an aqueous buffer and a non-UV active water miscible organic solvent. The effect of the organic and aqueous phase and the proportions in which they are mixed will affect the analysis of the drug molecule. Selection of the mobile-phase is dependent on the ionogenic nature of the analyte and the hydrophobicity of the analyte in the mixture respectively (Jain, *et al.*, 2012; Singh, 2013).

1.3.6.1 Choice of organic solvent

The organic solvent (modifier) is added to lower the polarity of the aqueous mobile phase. The lower the polarity of the mobile phase, the greater it is eluting strength in RP-HPLC. Although a large variety of organic solvents can be used in RP-HPLC, in practice only a few are routinely employed (Prathpa *et al.*, 2013).

Selection of the organic modifier could be viewed as relatively simple. The choice is among usually between acetonitrile, methanol and rarely tetrahydrofuran. Acetonitrile as an organic modifier may offer these variations due to the introduction of a dual retention mechanism. The viscosity of water/organic mixtures should be considered as an additional parameter in the selection of organic modifier. Acetonitrile/water mixtures show roughly 2.5 times lower viscosity than that of equivalent methanol/water eluents; this means that one can use 2.5 faster flow rates with acetonitrile as organic modifier and develop faster separation methods than those with methanol, modifier. Acetonitrile is not ionogenic and is not a hydrogen bonding agent, but its four electrons offer strong dispersive interactions that should also be taken into account in the solvent selection. Changing the type of organic eluent may have an effect on the resulting selectivity of the two species in a mixture. There is no definite way to predict if changing the type and concentration of the organic eluent will impart a difference in selectivity of the closely eluting species. The types of solvent that are recommended are pure acetonitrile, pure

methanol, and a mixture of acetonitrile/methanol. Sometimes, a small addition of THF (up to 5%v/v) or isopropanol to either acetonitrile or methanol may lead to changes in the selectivity. The adsorption of the organic eluent component on the stationary phase and the interactions of the eluent with the analyte molecules play a significant role in determining the resultant selectivity of the separation (Kaushal and Srivastava, 2010).

1.3.6.2 Choice of pH

pH plays an important role in achieving the chromatographic separations as it controls the elution properties by controlling the ionization characteristics (Prathpa *et al.*, 2013).

If analyte is ionizable, the proper mobile-phase pH must be chosen based on the analyte p*Ka* so, the target analyte is in one predominate ionization state: ionized or neutral. If possible, method development at both of these defined mobile-phase pH values is encouraged to maximize the potential gains that may be obtained in regard to selectivity. Alteration of the mobile-phase pH is one of the greatest effects causing simultaneous changes in retention and selectivity between critical pair of components. Analytes may be analyzed in their ionic form or neutral form. This may be dependent on the type of analysis that is required. If fast analysis is required, then analysis of the component in its ionized form may be acceptable provided that the desired resolution from the matrix components is achieved. Different approach may be attempted, if adequate resolution of the active form from its process related impurities/degradation products/excepients are not obtained. Then mobile-phase additives may be added to the mobile phase or the mobile phase pH may be adjusted so that the analyte may be analyzed in its neutral form in order to potentially enhance the selectivity/resolution between critical pairs of components.

The pH of mobile phase can be controlled by use of buffer that increases its capacity. Buffers that are selected should have a good buffering capacity at the specified mobile-phase pH. A buffer concentration in the range of 10 to 50 mM is adequate for most reversed-phase applications. However, sometimes the concentration of the buffer does lead to improvement of peak shape, presumably because the cation of the buffer suppresses silanophilic interactions of the protonated base with accessible ionized residual silanols. This concentration should also be low enough to avoid problems with precipitation when significant amounts of organic modifiers are used in the mobile phase and, in the case of phosphate buffers, low enough to minimize the abrasive effect on pump seals (Ravisankar *et al.*, 2014; Kaushal and Srivastava, 2010).

1.3.6.3 Choice of ion-pair reagent

Use of ion-pair reagent will depend on the nature of analyte, if it is simple ion; use of ion-pair reagent improves selectivity. Methanol provides better solubility for ion-pair reagent as well as buffer and salts. So, it may be preferred over acetonitrile and THF. For selecting proper ion-pair reagent, alkyl chain length must be considered. The longer is the chain, more hydrophobic the counter ion and therefore the greater retention due to equilibrium between counter ion and column adsorbent (Ravisankar *et al.*, 2014).

1.3.7 Choice of mode of separation

Isocratic, constant eluent composition means that equilibrium conditions in the column and the actual velocity of compounds moving through the column are constant; analyte-eluent and analyte-stationary-phase interactions are also constant throughout the whole run. This makes isocratic separations more predictable, although the separation power (the number of compounds which could be resolved) is not very high.

Gradient separation significantly increases the separation power of a system mainly because of the dramatic increase of the apparent efficiency. The condition where the tail of a chromatographic zone is always under the influence of a stronger eluent composition leads to the decrease of the peak width. Peak width varies depending on the rate of the eluent composition variation (gradient slope) (Gupta, *et al.*, 2012).

Gradient elution is employed for complex multicomponent samples since it may not be possible to get all components eluted between k (retention factor) 1 and 10 using a single solvent strength under isocratic conditions. This leads to the general elution problem where no one set of conditions is effective in eluting all components from a column in a reasonable time period while still attaining resolution of each component. This necessitates the implementation of a gradient. Employing gradients shallow or steep allows for obtaining differences in the chromatographic selectivity. This would be attributed to the different slopes of the retention versus organic composition for each analyte in the mixture. When a gradient method is used, the column must be allowed to equilibrate at the starting mobile-phase conditions prior to the next sample injection and the start of the next gradient run ((Rao *et al.*, 2015)

In deciding whether a gradient would be required or whether isocratic mode would be adequate, an initial gradient run is performed, and the ratio between the total gradient time and the difference in gradient time between the first and last components are calculated. If the calculated ratio is <0.25, isocratic is adequate; when the ratio is >0.25, gradient would be beneficial (Gupta, *et al.*, 2012).

1.3.8 Choice of temperature

Temperature can have a profound effect on RP-HPLC, especially for low molecular weight solutes. The viscosity of the mobile phase used in RP-HPLC decreases with increasing column temperature. Since mass transport of solute between the mobile phase and the stationary phase is a diffusion-controlled process, decreasing solvent viscosity generally leads to more efficient mass transfer and, therefore, higher resolution. Increasing the temperature of a reversed phase column is particularly effective for low molecular weight solutes since they are suitably stable at the elevated temperatures (Prathpa *et al.*, 2013).

1.3.9 Finalize the method

The final step in method development process is to evaluate actual samples, by the new or improved standard method, confirming the suitability for use. Experiments should be designed to challenge the test method. Evaluated the optimized method by testing actual samples. It may be important to determine if the test method can differentiate between products manufactured at varying processing conditions and whether the method is capable of measuring the differences in samples that have been altered slightly, make any final adjustments, to the procedure to address any problems encountered while evaluating actual samples.

The documented test method should be revised to include any changes made to the procedure during the finalization step or to add any additional information, which may be beneficial to the user. In addition to specific information on the requirements for instrumentation, operating conditions, steps of the procedure include any information which is critical to successfully apply the method. For instance there may be a very specific way to prepare the sample where the outcome is increased accuracy and precision (USP, 2000).

1.4 Stability indicating assay method development

A stability indicating assay method is a validated quantitative analytical method that can detect the changes with time in the chemical, physical, or microbiological properties of the drug substance and drug product, and that are specific so that the contents of active ingredient, degradation products, and other components of interest can be accurately measured without interferences. Forced degradation having an important role in the development of stability indicating assay method. A degradation product is a molecule resulting from a change in the active ingredient as a result of processing or storage. Compounds that formed from a reaction of the active ingredient with an excipients or

container closure component are considered degradation products (Riddhiben *et al.*, 2011; Rao *et al.*, 2015).

1.4.1 Techniques employed in stability indicating assay methods

The techniques involve in stability indicating assay methods include:

1.4.1.1 Titrimetric and spectrophotometric methods

In these methods, usually the objective is the analysis of the drug of interest alone in the matrix of excipients, additives, degradation products, impurities, and also other drugs in the case of the combination products. Their advantage is the low cost and simplicity, though sometimes they are not sensitive.

1.4.1.2 Chromatographic methods

Because of the requirement of separation of multiple components during analysis of stability samples, chromatographic methods have taken precedence over the conventional methods of analysis. Other than separation of multiple components, the advantage of chromatographic methods is that these possess greater accuracy and sensitivity for even small quantities of degradation products produced. Various chromatographic methods that have been used are thin-layer chromatography (TLC), high performance thin-layer chromatography (HPTLC), gas chromatography (GC), HPLC and capillary electrophoresis (CE).

1.4.1.3 Advanced techniques

Advanced stability indicating assay methods include the use of proton nuclear magnetic resonance (^{1}HNMR) spectroscopy. There are several hyphenated techniques which include LC-MS, LC-MS-MS (tandem mass spectroscopy), GC-MS, LC-NMR and CE-MS techniques for identity confirmation of known and unknown degradation products and their selective determination. LC-MS-MS is used to obtain molecular weight and fragmentation information and LC-NMR is used for determination of detailed structural information. The advantages of these methods are less time consuming and high sensitivity. By these methods several degradation products can be identified simultaneously (Bakshi and Singh, 2002; Jain *et al.*, 2010).

1.4.2 Stability indicating assay method development steps

The practical steps involved in the development of stability indicating assay method are the same as in HPLC method development with one additional step which is conducting a forced degradation study.

1.4.2.1 Stress (forced degradation) studies

Forced degradation or stress studies are undertaken to deliberately degrade the sample. These studies are used to evaluate an analytical method's ability to measure an active ingredient and its degradation products, without interference, by generating potential degradation products.

1.4.2.1.1 Objectives of forced degradation studies

Forced degradation studies are carried out to achieve the following purposes (Blessy *et al.*, 2014; Saimalakondaiah *et al.*, 2014).

- To determine degradation pathways of drug substance and drug products and to generate degradation profile.
- To discriminate degradation products in formulations related to drug substances versus those that are related to non-drug substances (excipients).
- To elucidate structure of degradation products.
- To determine the intrinsic stability of a drug substance molecule in solution and solid state.
- To reveal the thermolytic, hydrolytic, oxidative and photolytic degradation mechanism of the drug substance and drug product.
- To develop and validate a stability indicating method.
- To identify impurities related to drug substances or excepients.
- To understand the drug molecule chemistry.
- To generate more stable formulations.
- To solve stability-related problems.

1.4.2.1.2 Limits for degradation

Degradation of drug substances between 5% and 20% has been accepted as reasonable for validation of chromatographic assays. Some pharmaceutical scientists think 10% degradation is optimal for use in analytical validation for small pharmaceutical molecules for which acceptable stability limits of 90% of label claim is common. Others suggested that drug substance spiked with a mixture of known degradation products can be used to challenge the methods employed for monitoring stability of drug product. It is not necessary that forced degradation would result in a degradation product. The study can be terminated if no degradation is seen after drug substance or drug product has been exposed to stress conditions. This is indicative of the stability of the molecule under test. Over- stressing a sample may lead to the formation of a secondary

degradation product that would not be seen in formal shelf-life stability studies and under-stressing may not generate sufficient degradation products. Protocols for generation of product-related degradation may differ for drug substance and drug product due to differences in matrices and concentrations. It is recommended that maximum of 14 days for stress testing in solution (a maximum of 24 h for oxidative tests) to provide stressed samples for methods development (Prathpa *et al.*, 2013; Blessy *et al.*, 2014).

1.4.2.1.3 Selection of drug concentration

It is recommended that the studies should be initiated at a concentration of 1 mg/ml. By using drug concentration of 1 mg/ml, it is usually possible to get even minor decomposition products in the range of detection. It is suggested that some degradation studies should also be done at a concentration which the drug is expected to be present in the final formulations (Quadri *et al.*, 2014).

For drugs that have poor water solubility, stress studies can be conducted either in suspension or in solutions using organic co-solvents. Co-solvent selected include (Aubry *et al.*, 2009).

- DMSO, acetic acid, and propionic acid are useful for acidic conditions.
- DMSO, N-methylpyrrolidone(NMP), and acetonitrile (ACN) work under neutral conditions.
- Glyme and 1,4-dioxane facilitate reactions in base.
- ACN is the co-solvent of choice for photochemical reaction.
- Methanol is avoided for $-CO_2H$, amide, $-OH$, $ArNH_2$.

1.4.2.1.4 Strategy for selection of degradation conditions

The choice of stress conditions should be consistent with the product's decomposition under normal manufacturing, storage, and use conditions which are specific in each case. A minimal list of stress factors suggested for forced degradation studies must include acid and base hydrolysis, thermal degradation, photolysis, and oxidation.

1.4.2.1.4.1 Acid and base hydrolysis conditions

Hydrolysis is one of the most common degradation chemical reactions over a wide range of pH. Hydrolysis is a chemical process that includes decomposition of a chemical compound by reaction with water. Hydrolytic study under acidic and basic condition involves catalysis of ionizable functional groups present in the molecule. The functional groups likely to introduce acid/base hydrolysis are compounds that have labile carbonyl

functionality such as amides (lactams), esters (lactones), carbamates, imides, imines, alcohols and aryl amines. The selection of the type and concentrations of acid or base depends on the stability of the drug substance. Hydrochloric acid or sulfuric acids (0.1–1 M) for acid hydrolysis and sodium hydroxide or potassium hydroxide (0.1–1M) for base hydrolysis are suggested as suitable reagents for hydrolysis. If there is no degradation, elevated temperature (50°C–70°C) is applied. Stress testing should not exceed more than 7 days. The degraded sample is then neutralized using suitable acid, base or buffer, to avoid further decomposition (Shete *et al.*, 2014; Charde *et al.*, 2013).

1.4.2.1.4.2 Oxidation condition

Hydrogen peroxide is widely used for oxidation of drug substances in forced degradation studies but other oxidizing agents such as metal ions, oxygen, and radical initiators (azobisisobutyronitrile, AIBN) can also be used. Selection of an oxidizing agent, its concentration, and conditions depends on the drug substance. It is reported that subjecting the solutions to 0.1–3% hydrogen peroxide at neutral pH and room temperature for seven days or up to a maximum 20% degradation could potentially generate relevant degradation products. The oxidative degradation of drug substance involves an electron transfer mechanism to form reactive anions and cations. Amines, sulfides and phenols are susceptible to electron-transfer oxidation to give N-oxides, hydroxylamine, sulfones and sulfoxide. The functional group with labile hydrogen like benzylic carbon, allylic carbon, and tertiary carbon or α-positions with respect to hetro atom is susceptible to oxidation to form hydro peroxides, hydroxide or ketone (Aubry *et al.*, 2009; Saimalakondaiah *et al.*, 2014).

1.4.2.1.4.3 Photolytic condition

Exposure of drug molecules to light may produce photolytic degraded products. The rate of photo degradation depends upon the intensity of incident light and quantity of light absorbed by the drug molecule. Photolytic degradation is carried out by exposing the drug substance or drug product to a combination of visible and UV light. The most commonly accepted wavelength of light is in the range of 300-800 nm to cause the photolytic degradation. The photolytic degradation can occur through non oxidative or oxidative photolytic reaction. The non-oxidative photolytic reaction includes isomerization, dimerization, cyclization, rearrangements, decarboxylation and hemolytic cleavage of X-C heteros bonds, N-alkyl bond, SO_2-C bonds, etc., and while oxidative photolytic reaction occurs through either singlet oxygen or triplet oxygen mechanism.

The singlet oxygen reacts with the unsaturated bonds, to form photo oxidative degradation products; whereas triplet oxygen reacts with free radical of the drug molecule, which then reacts with a triplet oxygen molecule to form peroxide. Hence, light can also act as a catalyst to oxidation reactions (Saimalakondaiah *et al.*, 2014; Hotha *et al.*, 2013).

1.4.2.1.4.4 Thermal condition

In general, rate of a reaction increases with increase in temperature. Hence, the drugs are susceptible to degradation at higher temperature. Thermal degradation involves different reactions like pyrolysis, hydrolysis, decarboxylation, isomerization, rearrangement and polymerization. Effect of temperature on thermal degradation of a substance is studied through Arrhenius equation:

$K= A\ e^{-Ea/RT}$

Where k is specific reaction rate, A is frequency factor; Ea is energy of activation, R is gas constant and T is absolute temperature. Thermal degradation study is carried out at 40˚C to 80˚C. The most widely accepted temperature is 70˚C at low and high humidity for 1-2 months. High temperature (>80˚C) may not produce predictive degradation pathway. The use of high-temperatures in predictive degradation studies assumes that the drug molecule will follow the same pathway of decomposition at all temperatures (Saimalakondaiah *et al.*, 2014; Hotha *et al.*, 2013; Blessy *et al.*, 2014).

1.5 Validation

Method validation can be defined as establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics. Method validation is an integral part of the method development; it is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity, and potency of the drug substances and drug products (Bliesner, 2006; Shah *et al.*, 2012).

1.5.1 Need of validation

Validation is an expensive and time-consuming process. However, the use of a validated method eliminates testing repetitions and improves the prestige of the laboratory. Validation of a method must be performed for the following
reasons:

- Assuring high quality of the results.

- Reaching acceptance of the products by international agencies.
- Achieving the range of "official/reference method" approved by regulatory agencies.
- Conforming with mandatory requirement for accreditation of the laboratory by ISO guidelines.
- Obeying compulsory condition for registration of any pharmaceutical product or pesticide formulation.

1.5.2 Types of analytical procedure to be validated

The four most common types of analytical procedures are identification tests, quantitative measurements for impurities content, limit tests for the control of impurities, and quantitative measure of the active component in samples of drug substance or drug product or other selected component(s) in the drug product (Patil *et al.*, 2014; ICH, 2005; Reddy *et al.*, 2011).

Table (1.1) describes the recommended validation parameter should be evaluated for the common types of analytical procedures (Ederveen, 2010).

Table (1.1) Validation parameters for analytical procedures.

Type of analytical procedure	**Identification**	**Impurity test**		**Assay; content ;potency**
		Quantitation	**Limit**	
Accuracy	-	+	-	+
Precision				
Repeatability	-	+	-	+
Intermed. precision	-	+	-	+
Reproducibility	-	+	-	-
Specificity	+	+	+	+
Detection limit	-	+	+	-
Quantitation limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

- : not normally evaluated. + : normally evaluated.

1.5.3 Parameters for method validation

1.5.3.1 Specificity

The specificity of a method is its ability to measure accurately and specifically the analyte in the presence of components that are expected to be present in the sample matrix such as starting materials, intermediates in the synthesis, and inactive ingredients in the formulated products, and the degradation products. A method can be 'specific' for one or more components of a mixture, but 'non-specific' for another component (Kazusaki *et al.*, 2012).

1.5.3.3 Limit of detection and limit of quantification

Limit of detection (LOD) is the lowest level of analyte that can be detected, but not necessarily quantitated as an exact value.

Limit of quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. LOD and LOQ are usually expressed in terms of a concentration of analyte in the sample. Several approaches for establishing the LOD and LOQ are possible, depending on whether the procedure is non instrumental or instrumental.

The first approach is expressed as the concentration at specified signal – to – noise ratio. ICH guidelines have recognized 3:1 ratio for LOD and 10:1 ratio for LOQ.

The second approach is based on SD of the response and the slope (s) of the calibration curve as:

LOD = 3.3 × (SD/s), and LOQ = 10 × (SD/s).

SD of the response can be determined based on the SD of the blank, on the residual SD of the regression line or the SD of y- intercept of regression line.

The third approach is to determine LOD and LOQ visually and this is for non instrumental methods (Shabir, 2003).

1.5.3.3 Linearity

Linearity of an analytical procedure is the ability (within a given range) to obtain test results of variable data (e.g., absorbance and area under the curve) which are directly proportional to the concentration (amount of analyte) in the sample.

Linearity is usually demonstrated directly by dilution of a standard stock solution. It is recommended that linearity be performed by serial dilution of a common stock solution (Chan, 2004; Araujo, 2009).

1.5.3.4 Range

The range of an analytical method is the interval between the upper and lower levels that have been demonstrated with precision, accuracy and linearity using the analytical method. So, the acceptable range will be defined as the concentration interval over which linearity, accuracy and precision are acceptable (Swartz and Krull, 2012).

1.5.3.6 Accuracy

Accuracy is the closeness of the test results obtained by the analytical method to the true value. Accuracy is usually determined in one of four ways. First, accuracy can be assessed by analyzing a sample of known concentration (reference materials) and comparing the measured value to the true value. The second approach is to compare test results from the new method with results from an existing alternate well-characterized procedure that is known to be accurate. The third approach, based on the recovery of known amounts of analyte, is performed by spiking analyte in blank matrices. The fourth approach is the technique of standard additions, which can also be used to determine recovery of spiked analyte (Ermer, 2005).

1.5.3.6 Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple samples of the same homogeneous sample under prescribed conditions. Precision is usually investigated at three levels: repeatability, intermediate precision, and reproducibility.

Repeatability is a measure of the precision under the same operating conditions over a short interval of time. It is sometimes referred to as intra-assay precision. It should be determined from a minimum of nine determinations covering the specified range for the procedure (three levels, three repetition each or from a minimum of six determinations at 100% of the test concentration). The standard deviation, relative standard deviation (coefficient of variation), and confidence interval should be reported as required by the ICH.

Intermediate Precision is defined as the variation within the same laboratory. The extent to which intermediate precision needs to be established depends on the circumstances under which the procedure is intended to be used. Typical parameters that are investigated include day-to-day variation, analyst variation, and equipment variation. ICH recommended the reporting of standard deviation, relative standard deviation (coefficient of variation), and confidence interval of the data.

Reproducibility measures the precision between laboratories as in collaborative studies. This parameter should be considered in the standardization of an analytical procedure (Geetha *et al.*, 2012).

1.5.3.7 Robustness

The robustness of a method is its ability to remain unaffected by small deliberate variations in method parameters. The robustness of a method is evaluated by varying method parameters such as percent organic solvent, pH of buffer in mobile phase, ionic strength, different HPLC columns (lots and/or suppliers), column temperature, flow-rate etc. These method parameters may be evaluated one factor at a time or simultaneously as part of a factorial experiment (Shabir, 2003; Chan, 2004). Typical variation include under validation process , flow rate 10 %, wavelength 2 nm, mobile phase composition, generally organic composition 2 or 5%, temperature 5% and pH of the mobile phase 0.2 unit (Okafo and Robert, 2003).

1.6 Amlodipine besylate

Amlodipine besylate is the besylate salt of amlodipine, it's chemically described as 3-Ethyl-5-methyl (±)-2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylate, monobenzenesulphonate. It is molecular formula is $C_{20}H_{25}ClN_2O_5 \bullet C_6H_6O_3S$, and its structural formula as in Figure (1.1).

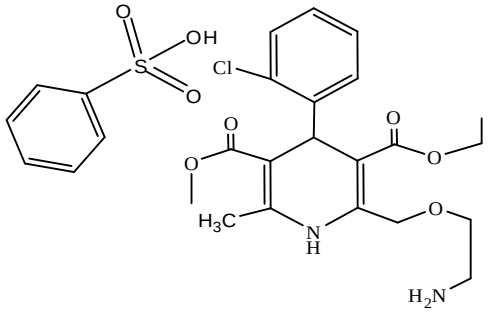

Figure (1.1) Structural formula of amlodipine besylate

Amlodipine besylate is a white crystalline powder with a molecular weight of 567.1. It is slightly soluble in water and sparingly soluble in ethanol. Amlodipine besylate tablets are formulated as white tablets equivalent to 2.5, 5 and 10 mg of amlodipine for oral administration. In addition to the active ingredient, amlodipine besylate, each tablet contains the following inactive ingredients: microcrystalline cellulose, dibasic calcium phosphate anhydrous, sodium starch glycolate, and magnesium stearate (Alsarra, 2009; Momin *et al.*, 2012).

1.6.1 Pharmacodynamics

Amlodipine is a dihydropyridine calcium antagonist, that inhibits the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle. Experimental data suggest that amlodipine binds to both dihydropyridine and nondihydropyridine binding sites. Amlodipine inhibits calcium ion influx across cell membranes selectively, with a greater effect on vascular smooth muscle cells than on cardiac muscle cells. Within the physiologic pH range, amlodipine is an ionized compound (pKa = 8.6), and its kinetic interaction with the calcium channel receptor is characterized by a gradual rate of association and dissociation with the receptor binding site, resulting in a gradual onset of effect. Amlodipine is a peripheral arterial vasodilator that acts directly on vascular smooth muscle to cause a reduction in peripheral vascular resistance and reduction in blood pressure (Reddy *et al.*, 2013; Saravanan *et al.*, 2010).

1.6.2 Pharmacokinetic and metabolism

1.6.2.1 Absorption

Orally administered amlodipine is slowly and almost completely absorbed from the gastrointestinal tract. Although the time to reach peak plasma concentration is 6 – 9 hours, the drug has a high bioavailability (64% – 90%). When undergoing absorption, the drug molecule orients itself between the hydrophobic and hydrophilic regions of the membrane. The ionized side chain interacts with the negatively charged region of the phospholipids' head, and this slows diffusion of amlodipine through the membrane to its receptor site. This interaction accounts for several of the drug's unique pharmacokinetic parameters, including its gradual onset and offset of action (Nayler, 1993).

1.6.2.2 Metabolism

Amlodipine is extensively (about 90%) converted to inactive metabolites via hepatic metabolism via CYP 3A4. Metabolism studies have indicated that oxidation to the pyridine analog is the major pathway of metabolism (Beresford *et al.*, 1998).

1.6.2.3 Excretion

The majority of amlodipine dose (10% of the parent compound and 60% of the inactive metabolites) is excreted in the urine and feces. The terminal elimination half-life of amlodipine ranges from 30 - 50 hours (Nayler, 1993).

1.6.3 Methods of analysis

Several methods were reported for the estimation of amlodipine besylate alone and in combination with other drugs in pharmaceutical preparations and biological fluids

including: HPLC, HPTLC, LC-MS, spectrofluorometry and spectrophotometry (Jampana *et al.*, 2014; Sah and Arora, 2012; Ansari *et al.*, 2012).

The official USP method used isocratic elution with mobile phase comprises 35% methanol, 5% acetonitrile and 50% buffer solution of pH 3 which is prepared from triethylamine adjusted with phosphoric acid. The column used is 3.9 mm × 15 cm packed with Octadecylsilane chemically bonded to porous silica with 10 µm diameter, using 1.0 ml/min flow rate, and detection is at 237nm in ambient temperature. Retention time is about 12.402 min. BP also describe isocratic HPLC method for the assay of amlodipine in raw material.

1.7 Esomeprazole magnesium

Esomeprazole Magnesium (as trihydrate) belongs to the group of proton pump inhibitors (PPI). It is the enantiomer of omeprazole. Chemically it is 5-Methoxy-2- (S) [(4-methoxy-3, 5-dimethyl-2-pyridinyl) methyl] sulfinyl]-1H-benzimidazole magnesium salt trihydrate with molecular formula$C_{17}H_{18}N_3O_3S•3H_2O$ •Mg and its structural formula as in Figure (1.2).

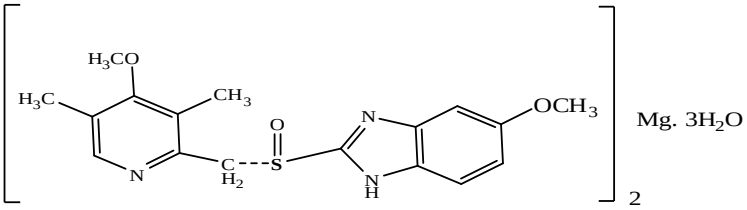

Figure (1.2) Structural formula of esomeprazole magnesium

Esomeprazole is slightly soluble in water and freely soluble in methanol. It is white to slightly colored crystalline powder, containing three water molecules of hydration with molecular mass of 767.2 g/mol and melting point of 155°C. The pKa of benzimidazole (esomeprazole base) is 8.8. The stability of esomeprazole magnesium is a function of pH; it rapidly degrades in acidic media, but it has acceptable stability under alkaline conditions (Kulkarni *et al.*, 2011; Patel *et al.*, 2014).

1.7.1 Pharmacodynamics

Esomeprazole suppresses gastric acid secretion from gastric parietal cells. Esomeprazole is a weak base that is concentrated in the acidic environment of parietal cells and converted to the active inhibitor, a chiral sulphonamide. This inhibitor binds irreversibly to specific cysteines, resulting in an inhibition of H^+/K^+-ATPase enzyme activity.

Inhibition of gastric acid secretion is dose dependent, being observed in the range of 20–40 mg/day (Al-Judaibi *et al.*, 2010; Mckeage *et al.*, 2008).

1.7.2 Pharmacokinetic and metabolism

1.7.2.1 Absorption

Esomeprazole is absorbed rapidly after oral administration. The peak serum concentration of esomeprazole (Cmax) was found to be within 0.5 hours of ingestion of an oral solution containing 20 mg and within 1 to 3.5 hours for encapsulated enteric-coated granules (40 mg). The bioavailability of the drug is 64% and is highly dependent on food intake, which delays and decreases absorption but does not appear to have an effect on intragastric acidity (Islam *et al.*, 2011; Al-Judaibi *et al.*, 2010).

1.7.2.2 Metabolism

Esomeprazole is extensively metabolized in the liver by the cytochrome P450 (CYP) enzyme system. The metabolites of esomeprazole lack antisecretory activity. The major part of esomeprazole's metabolism is dependent upon the CYP2C19 isoenzyme and CYP3A4, which form two main metabolites, esomeprazole sulphone and 5-hydroxy esomeprazole, both pharmacologically inactive (Scott *et al.*, 2002; Medhi *et al.*, 2012; Hassan-Alin *et al.*, 2000).

1.7.2.3 Excretion

Approximately 80% of an oral dose of esomeprazole is excreted as inactive metabolites in the urine, and the remaining is found as inactive metabolites in the feces; less than 1% of the parent compound is found in the urine (Mckeage *et al.*, 2008).

1.7.3 Methods of analysis

Literature survey reveals that several analytical techniques have been described for the determination of esomeprazole in bulk, pharmaceutical formulations biological samples which include HPLC, HPTLC, and spectrophotometry (pasumarthi et al., 2014; Mahaparale and Deshpande, 2015; Gupta, Mishra, et al., 2012).

The official USP method used isocratic elution with mobile phase comprises 35% acetonitrile and 65% buffer solution of pH 7.6 which is prepared from monobasic sodium phosphate and dibasic sodium phosphate. The column used is 4.6 mm × 15 cm packed with Octadecylsilane chemically bonded to porous silica with 10 µm diameter, using 1.0 ml/min flow rate, and detection is at 280nm in ambient temperature. Retention time is about 7.68 min. BP also describe isocratic HPLC method for the assay of esomeprazole in raw material.

1.8 Aims and objectives

(I) General objectives

To design and validate chromatographic and spectrophotometric stability indicating methods for the determination of amlodipine besylate and esomeprazole magnesium.

(II) Specific objectives

- To assess the proposed methods by full validation protocol according to the ICH guidelines.
- To design proposed HPLC method to be applied as stability indicating method to be used in routine stability studies of both drugs even in presence of their degradation products.
- To study the stability of amlodipine and esomeprazole drugs under the effect of temperature, UV light, acids, bases, oxidation, and exposure to sun light.
- To study the kinetic of degradation in order to determine the order of the reaction, rate constant, and half life ($t_{(1/2)}$) in all conditions that affect the stability of both drugs.
- To study the effect of separately added excepients, which are commonly, used in pharmaceutical dosage forms on the thermal stability of both drugs.

2. MATERIALS, INSTRUMENTS AND METHODS

2.1 Materials

2.1.1 Chemical solvents

Methanol HPLC grade from Duksan pure chemicals Korea.

Acetonitrile HPLC grade from Fisher scientific limited UK.

Hydrochloric acid and acetic acid from Sigma Aldrich labor chemi kalien.

Hydrogen peroxide from DAEJUNG chemicals and metals Co Ltd, Germany.

Chloroform HPLC/Spectro grade from Tedia company Inc, USA.

Ethanol absolute duty free HPLC grade from fisher scientific limited, UK.

Triethylamine from Sigma .Aldrich chemie, Switherland.

2.1.2 Chemical reagents

Amlodipine besylate (99.66 %) and esomeprazole magnesium (99.72 %) are from Amipharma Laboratories Ltd, Khartoum, Sudan.

Mangessium stearate from Calmags.BmbH, India.

Microcrystalline cellulose from JSR pharma, India.

Starch maze from Roquette, India.

Talc powder. BP.IMERYS tall, India.

Dibasic calcium phosphate from British drug hose, England.

Sodium starch glycolate from JPT, India.

Methyl orange, potassium dihydrogen phosphate, sodium acetate, and sodium hydroxide from Daejung chemicals and metals Co., Ltd, Germany.

Eriochrome black T from Fisher scientific limited, UK.

Ammonium acetate, potassium acetate, and nicotineamide are from Aldrich Chemical Co., St. Louis, USA.

Alizain red s from Riedel-de Hanen AG, Germany.

2.2 Instruments

2.2.1 High-Performance Liquid Chromatograph

The following HPLCs were used:

a) High-performance liquid chromatograph, Water 2695 separation module USA consisting of the following items:

1-Photodiode array detector waters 2996 .

2-Injector loop size up to 100 µl.

3-Empowere software 2.

4. Autosampler capacity of 120 vial.

b) High-performance liquid chromatograph Shimadzu Japan, consisting of the following items.

1. Auto sampler SIL-20A.

2. Pumps (RHPLC) Quaternary pump serial number LC 10 At VP.

3. Column oven CTO – 10 AS VP.

4 UV- VIS detector SPD – 10AVP

5- Software LC solution.

6- Injection loop up to 100 µL.

2.2.2 Ultraviolet visible spectrophotometer

The following UV-VIS spectrometers were used:

a) UV-1800, double beam, wavelength 190-1100nm-SHIMADZU Japan.

b)Thermonicolet evolution 300, wavelength 190-1100nm, USA.

2.2.3 Infrared Spectrometer

Infrared spectrometer, Bruker, UK.

2.2.4 General equipments

pH meter Hanna instruments, HI 255 combined meter (pH/ mV and EC/TDS/NaCl).

Water bath Bushi heating bath B490, Germany.

Analytical balance, KERN ALS s max 120gm, drifting 0.1mg Germany.

Elmasonic E 30 H , D-78224, Germany.

UVITECH, LF – 204-Ls (365 nm and 254 nm tubes), M032693, Germany.

2.3 Methods

2.3.1 Identification test for Amlodipine besylate and esomeprazole magnesium

A standard IR test was done for both drugs using Bruker instrument. The scan was made in the range ($500cm^{-1}$- $4000\ cm^{-1}$) with total of ten scans.

2.3.2 Spectrophotometric method development

2.3.2.1 Effect of solvent on UV spectrum of amlodipine besylate and esomeprazole magnesium

10 mg of each amlodipine besylate and esomeprazole magnesium were weighed and transferred into separate 100 ml-volumetric flask, then dissolved in methanol, ethanol, chloroform, acetonitrile, and propanol and diluted to the mark with the same solvent. An aliquot of 5 ml of those solutions was transferred into 10-ml-volumetric flask and diluted to the mark with the same solvent to obtain a solution of 50 µg/ml, and then the solution was scanned over the range from 200 nm to 400 nm.

2.3.2.2 Spectrophotometric method development for determination of amlodipine besylate

2.3.2.2 .1Optimization of the methods

2.3.2.2.1.1 Method 1

2.3.2.2.1.1.1 Choice of solvent

Solubility of 100 mg amlodipine besylate was checked in 50 ml of different aqueous hydrotropic agent solutions: 0.5 M potassium acetate, 0.5 M ammonium acetate, 0.5 M nicotineamide, 1.0 M sodium citrate, 1.0 M ascorbic acid, and 1.0 M sodium salicylate. The solution containing the two solutes was shaken vigorously for 10 min.

2.3.2.2.1.1.2 Preparation of reagents

0.5 M solution of each ammonium acetate, potassium acetate, and nicotineamide were prepared by dissolving 3.853, 4.908, and 6.106 g of the respective reagent and diluting each solution to the mark in 100 ml-volumetric flask with distilled water.

2.3.2.2.1.1.3 Preparation of standard solution

A stock solution of 200 µg/ml was prepared by dissolving 20 mg of amlodipine besylate standard in 50 ml of 0.5M hydrotropic agent, sonicated for 20 min and diluted to the mark in 100 ml-volumetric flask with distilled water.

2.3.2.2.1.1.4 Preparation of sample solution

20 tablets of amlodipine besylate (10 mg/tablet) were weighed and finely powdered. A portion of the powder equivalent to 10 mg of drug was dissolved in 50 ml of hydrotropic agent solution, sonicated for 20 min, filtered and diluted to the mark in 100 ml-volumetric flask with distilled water to give a solution of 100 µg/ml.

2.3.2.2.1.1.5 Procedure for calibration curve

Serial concentrations were prepared from the stock solution by taking 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, and 4.5 ml into 10 ml-volumetric flasks and diluting to the mark with distilled water. Absorbances of these solutions were measured at λ_{max} 365 nm against blank solution treated in the same way omitting only the drug.

2.3.2.2.1.2 Method 2

2.3.2.2.1.2 .1Choice of solvent

Amlodipine besylate was found to be soluble in methanol after studying its solubility in different solvents.

2.3.2.2.1.2 .2 Preparation of reagents solution

A stock solution of 0.01 % (w/v) aqueous methyl orange was prepared by dissolving 0.01 g of methyl orange in distilled water and the volume was madeup to 100 ml. Acetate buffer was prepared by dissolving 6.81 g of anhydrous sodium acetate in 100 ml distilled water; pH was adjusted to 3.5 ± 0.01 using 1 M Acetic acid.

2.3.2.2.1.2 .3 Preparation of standard solution

Stock solution (50 µg/ml) was prepared by dissolving 5 mg of amlodipine besylate standard in 2 ml of methanol and diluted to the mark in 100 ml-volumetric flask with distilled water.

2.3.2.2.1.2.4 Preparation of sample solution

20 tablets of amlodipine besylate (10 mg/tablet) were weighed and finely powdered. A portion of the powder equivalent to 5 mg of the drug was dissolved in 2 ml of methanol, diluted with distilled water, sonicated for 20 min, filtered and diluted to the mark in 100 ml-volumetric flask with distilled water to obtain a solution of 50 µg/ml.

2.3.2.2.1.2 .5 Optimization of the factors affecting the reaction

2.3.2.3.1.2 .5.1 Effect of volume of methyl orange

The effect of volume of methyl orange was studied in the range from 0.5 to 3 ml.

2.3.2.2.1.2 .5.2 Effect of pH

The effect of pH of the buffer solution was studied in the range from 2 to 4.5.

2.3.2.2.1.2 .5.3 Effect of volume of buffer

The effect of volume of buffer was studied in the range from 0.25 to 2 ml.

2.3.2.2.1.2.6 Procedure for calibration curve

0.5 ml of drug solution (50 µg/ml) was transferred into a 50 ml-separating funnel. 1.0 ml of acetate buffer solution (pH 3.5) and 2.0 ml of 0.01 % methyl orange solution were added to the separating funnel. The volume was adjusted to 10 ml with distilled water and mixed well. 10 ml of chloroform was added to the separating funnel, and the content of separating funnel was shaken vigorously for 3 min. The two phases were allowed to stand for clear separation and the chloroform layer was separated. Absorbance of the organic layer was recorded at 427 nm against blank treated in the same way omitting the drug. The procedure was repeated using 1, 2, 3, 4, 5, and 6 ml of drug solution.

2.3.2.2.1.2.7 Stoichiometry of the reaction

Stock solutions of equimolar solution of 7.5×10^{-5} M of each methyl orange and amlodipine besylate were prepared in distilled water. A series of solutions were prepared in which the total volume of the drug and reagent was kept at 7 ml. The reagents were mixed in various proportions and madeup to 10 ml with distilled water, following the above-mentioned procedure in section 2.3.2.3.1.2.6.

2.3.2.2.2 Validation of spectrophotometric methods

2.3.2.2.2.1 Linearity and range

Linearity and range were investigated according to the procedure for calibration curve.

2.3.2.2.2.2 Precision

Precision for method 1 was assessed using standard solution of concentrations 10, 20, and 30 µg/ml prepared by taking the respective volumes of 0.5, 1, and 1.5 ml from the stock solution (section 2.3.2.3.1.1.3) into 10 ml-volumetric flask and diluting to mark with distilled water. The absorbance was then recorded at three different times on the same day and also at different days (day 1, day 2, and day 3).

For method 2, standard solution of concentrations 6, 8, and 12 µg/ml were prepared by taking the respective volumes 1.2, 2.4, and 3.6 ml from the stock solution (section 2.3.2.3.1.2.6) into separating funnel following the described procedure in the same section. The absorbance was then recorded a three different times on the same day and also at different days (day 1, day 2, and day 3).

2.3.2.2.2.3 Recovery

Recovery for method 1 was determined using three sample solutions of concentrations 5, 10, and 20 µg/ml prepared by taking the respective volumes 0. 5, 1, and 2 ml from the

sample solution (prepared in section 2.3.2.3.1.1.4) into 10 ml-volumetric flask and diluting the volume to the mark using distilled water.

For method 2, it was done by using 10, 15, and 20 µg/ml sample solutions prepared by taking the respective volumes 2, 3, and 4 ml from sample solution (prepared in section 2.3.2.3.1.2.4) into separating funnel and following the procedure in section 2.3.2.3.1.2.6.

2.3.2.2.2.4 Robustness

Robustness was examined using a solution of concentration of 10 µg/ml for both methods and applying the same procedure as in section 2.3.2.3.1.1.5, and 2.3.2.3.1.2.6 for method 1 and 2, respectively with some variation in methods variables. For method 1, these variations included changing λ_{max} from 365nm to 363 and 367 nm, and changing the concentration of the hydrotropic agent solution from 0.5 M to 0.4 and 0.6 M. For method 2, the variations included changing λ_{max} from 427 nm to 425 and 429 nm, changing pH from 3.5 to 3.3 and 3.7 and changing methyl red concentration from 0.01% to 0.005 and 0.015 %.

2.3.2.3 Spectrophotometric method development for determination of esomeprazole magnesium

2.3.2.3 .1Optimization of the methods

2.3.2.3.1.1 Method 1

2.3.2.3.1.1 .1Choice of solvent

Esomeprazole magnesium was found to be soluble in methanol after studying its solubility in different solvents.

2.3.2.3.1.1.2 Preparation of reagents

Stock solution of 0.2 % (w/v) was prepared by dissolving 0.2 g of Alizarin red s in methanol and madeup the volume to 100 ml with the same solvent.

2.3.2.3.1.1.3 Preparation of standard solution

Stock solution of 200 µg/ml was prepared by dissolving 20 mg in methanol and madeup the volume to 100 ml with methanol.

2.3.2.3.1.1.4 Preparation of sample solution

10 tablets of esomeprazole (40 mg/tablet) were weighed and finely powdered. A portion of the powder equivalent to 10 mg of the drug was dissolved in methanol, sonicated for 20 min, filtered and diluted to the mark in 100 ml-volumetric flask with methanol to give a solution of 100 µg/ml.

2.3.2.3.1.1 .5 Optimization of the factors affecting the reaction

2.3.2.3.1.1.5.1 Effect of Volume of 0.2 % Alizarin red S

The effect of volume of Alizarin red S was studied in the range from 0.5 to 3 ml.

. 2.3.2.3.1.1.5.2 Effect of temperature

The influence of temperature was studied in the range from 30 to 50 ºC.

2.3.2.3.1.1 .5.3 Effect of heating time

In order to obtain the highest and stable absorbance, the effect of heating time was checked out on a water bath at 40 ± 1 ºC for periods ranging between 5 and 25 min.

2.3.2.3.1.1.6 Procedure for calibration curve

1 ml of 200 µg/ml of esomeprazole standard solution was transferred into 10 ml-volumetric flask, 2 ml of 0.2 % Aliz was added and the volume was madeup to 10 ml with methanol. The solution was heated on water bath at 40℃ for 15 min. The solution was cooled to room temperature and absorption was recorded at 521 nm against a reagent blank, treated similarly omitting the standard. The procedure was repeated with 0.25, 0.5, 1.5, 2, 2.5, 3, 3.5, and 4 ml of standard solution.

2.3.2.3.1.1.7 Stoichiometry of the reaction

Stock solutions of equimolar of 2.5×10^{-3} M of each Alizarin red s and esomeprazole were prepared in methanol. A series of 10 ml portions of the stock solution of esomeprazole and Alizarin were madeup comprising different complementary proportions (1:7 – 7:1 inclusive) in 10 ml-volumetric flask. The solutions were further manipulated as generally described in the recommended procedure in section 2.3.2.3.1.1.6.

2.3.2.3.1.2 Method 2

2.3.2.3.1.2 .1Choice of solvent

After studying the solubility of esomeprazole magnesium, methanol was chosen as a solvent.

2.3.2.3.1.2.2 Preparation of reagents solution

A stock solution of 0.5 % (w/v) eriochrome black T (EBT) was prepared by dissolving 0.5 g of EBT. in distilled water and the volume was madeup to 100 ml.

Acetate buffer was prepared by dissolving 6.81 g of anhydrous sodium acetate in 100 ml distilled water. pH was adjusted to 3.00 ± 0.01 using 1 M Acetic acid.

2.3.2.3.1.2.3 Preparation of standard solution

Stock solution of 100 µg/ml was prepared by dissolving 10 mg of esomeprazole in 2 ml of methanol and the volume was diluted to the mark in 10 ml-volumetric flask with distilled water.

2.3.2.3.1.2.4 Preparation of sample solution

10 tablets of esomeprazole (40 mg/tablet) were weighed and finely powdered. A portion of the powder equivalent to 10 mg of the drug was dissolved in 2 ml of methanol and diluted with water, sonicated for 20 min, filtered and diluted to the mark in 100 ml-volumetric flask with distilled water to form a concentration of 100 µg/ml.

2.3.2.3.1.2.5 Optimization of the factors affecting the reaction

2.3.2.3.1.2.5.1 Effect of Volume of 0.5 % EBT

The effect of volume of EBT was studied in the range from 1 to 3 ml.

2.3.2.3.1.2.5.2 Effect of pH

The effect of pH of the buffer solution was studied in the range from 2 to 4.

2.3.2.3.1.2.5.3 Effect of volume of buffer

The effect of volume of buffer was studied in the range from 0.25 to 1.5 ml.

2.3.2.3.1.2.6 Procedure for calibration curve

0.2 ml of 50 µg/ml of drug solution was transferred into a 50 ml-separating funnel. 2 ml of 0.5% EBT solution and 0.5 ml of buffer solutions of pH 3 were added to the separating funnel. The volume was adjusted to 10 ml with distilled water and mixed well. 10 ml of chloroform was added to the separating funnel and the content of separating funnel was shaken vigorously for 3 min. The two phases were allowed to stand for clear separation and the chloroform layer was separated. Absorbance of the organic layer was recorded at 511 nm against blank treated in the same way omitting the drug. The procedure was repeated using 0.6, 1, 2, 3, 3, 5, 6 and 7 ml of drug solution.

2.3.2.3.1.2.7 Stoichiometry of the reaction

Stock solutions of equimolar solution of 6.5×10^{-5} M of each EBT and esomeprazole were prepared separately in water. A series of solutions were prepared in which the total volume of the drug and reagent was kept at 6 ml. The volume of the drug and reagent were mixed in various proportions and madeup to 10 ml with distilled water, following the above-mentioned procedure in section 2.3.2.3.1.2.6.

2.3.2.3.2 Validation of spectrophotometric methods

2.3.2.4.2.1 Linearity and range

Linearity and range of the methods were investigated according to the procedure for calibration curve.

2.3.2.3.2.2 Precision

Precision for method 1 was assessed using standard solution of concentrations 15, 25, and 35 µg/ml prepared by taking respective volumes of 0.75, 1.25, and 1.75 ml from the stock solution (section 2.3.2.4.1.1.3) into 10 ml-volumetric flask following the procedure described in section 2.3.2.4.1.1.6; then the absorbances were measured at three different times on the same day and also at different days (day 1, day 2, and day 3).

For method 2, the precision was assessed using standard solution of concentrations 7, 14, and 21 µg/ml prepared by taking the respective volumes of 1.2, 2.4, and 3.6 ml from the stock solution (section 2.3.2.4.1.2.3) into separating funnel, following the procedure described in the same section 2.3.2.4.1.2.6; then the absorbances were measured at three different times on the same day and also at different days (day 1, day 2, and day 3).

2.3.2.3.2.3 Recovery

Recovery for method 1 was determined using three sample solutions of concentration 30, 40, and 50 µg/ml prepared by taking the respective volumes of 3, 4, and 5 ml of the sample solution prepared as described in section 2.3.2.4.1.1.4 into 10 ml-volumetric flask and following the procedure described in section 2.3.2.4.1.1.6.

For method 2 it was done by using 10, 15, and 20 µg/ml sample solutions prepared by taking the respective volumes of 1, 1.5, and 2 ml from sample solution (prepared as described in section 2.3.2.3.1.2.4) to separating funnel and following the procedure described in section 2.3.2.3.1.2.6.

2.3.2.3.2.4 Robustness

Robustness was examined using a solution of concentration of 10 µg/ml for both methods and applying the same procedure described in section 2.3.2.4.1.1.6 and 2.3.2.4.1.2.6 for method 1 and 2, respectively, with some variation in methods variables.

For method 1, these variations included changing λ_{max} from 521 nm to 519 and 523 nm, and changing the heating time from 15 min to 13 and 17 min.

For method 2, the variations included changing λ_{max} from 511 nm to 509 and 513 nm, and changing pH from 3 to 2.8 and 3.2.

2.3.3 HPLC method development and validation

2.3.3.1 HPLC method for the assay of amlodipine besylate: method 1

2.3.3.1.1 Optimization of method 1

Factorial design approach was used for optimization of chromatographic conditions for assay of amlodipine besylate. It was 2^3 full factorial design consisting of three factors; each factor had two levels. The three factors which affect the separation were percentage of organic modifier, acetonitrile(X_1), percentage of water (X_2), and flow rate (X_3). The levels of each factor are shown in Table (2.1).

Table (2.1) Levels of factors employed in factorial design.

Factor	Level	
	+1	-1
Acetonitrile %	60	55
Water %	40	35
Flow rate (ml/min)	1.5	1

Eight runs were performed to determine the optimum conditions for the chromatographic separation. The matrices of the eight runs are shown in Table (2.2).

Table (2.2) Matrices of 2^3 factorial optimization of mobile phase conditions for amlodipine HPLC method 1.

Exp. No.	Acetonitrile % (X_1)	Water % (X_2)	Flow rate (ml/min) (X3)
1	55	35	1.0
2	60	35	1.0
3	55	40	1.0
4	60	40	1.0
5	55	35	1.5
6	60	35	1.5
7	55	40	1.5
8	60	40	1.5

2.3.3.1.2 Preparation of mobile phase and diluent

Mobile phase, consisting of 60% acetonitrile, 40% water, and 0.1% triehylamine, was prepared, filtered and degassed before it was used.

The diluent was prepared by mixing 60% acetonitrile and 40% water.

2.3.3.1.3 Preparation of standard solution

A stock solution of 50 µg/ml amlodipine besylate standard was prepared by dissolving 10 mg of the standard in the diluents, and the volume was madeup to 200 ml with the diluent.

2.3.3.1.4 Preparation of sample solution

20 tablets of amlodipine besylate (10 mg/tablet) were weighed and finely powdered. A portion of the powder equivalent to 10 mg of the drug was dissolved in the diluent, sonicated for 20 min, filtered and madeup to 100 ml to obtain a solution of 100 µg/ml.

2.3.3.1.5 Selectivity

A placebo blank containing 10 mg of microcrystalline cellulose, dibasic calcium phosphate, sodium starch glycolate, and magnesium stearate was prepared in 100 ml-volumetric flask using the diluent, then diluted to obtain a concentration of 10 µg/ml.

2.3.3.1.6 System suitability

System suitability was checked using a solution of 50% concentration which was injected six times.

2.3.3.1.7 Linearity and range

Serial concentrations from the stock solution of amlodipine standard were prepared by diluting 0.5, 1, 2, 4, 6, and 8 ml to 10 ml using diluent.

2.3.3.1.8 Precision

Precision was assessed using three solutions prepared from the stock standard solution, and then the three solutions were analyzed three times on the same day and also at three different days in order to determine intra-day and inter-day precision, respectively.

2.3.3.1.9 Recovery (accuracy)

Recovery was performed in three levels. Three different concentrations of mixed standard and tablet sample solution were prepared to obtain known concentrations. The recovery was calculated from calibration curve.

2.3.3.1.10 Robustness

Robustness of the method was checked by varying the factors affecting the method each one at a time, and the change in response was observed. The factors which were studied in this method are shown in Table (2.3).

Table (2.3) Method robustness with various parameters.

Mobile phase composition			Flow rate (ml/min)			Wavelength of detection (nm)		
Level	Used	Original	Level	Used	Original	Level	Used	Original
+1	61:39	60:40	+1	1.1	1.0	+1	364	362
0	60:40		0	1.0		0	362	
-1	59:41		-1	0.9		-1	360	

2.3.3.1.11 Preparation of solutions for degradation study

2.3.3.1.11.1 Acid base degradation

25 mg of amlodipine besylate was weighed and transferred into 100 ml-volumetric flask, dissolved in 10 ml of methanol and sonicated for 10 min. 10 ml of 1 M of each sodium hydroxide and hydrochloric acid were added separately. The solutions were heated on water bath at 80° C for 1 hour, cooled to room temperature, neutralized to pH 7 using 0.5 M sodium hydroxide or hydrochloric acid; the volume was then diluted to the mark with the diluent. These solutions were filtered by using 45 µm filter. 5 ml of each solution was diluted to 25 ml using the diluent, and then it was analyzed using HPLC. The acidic- and alkaline-forced degradations were carried out in the dark in order to prevent the possible effect of light.

2.3.3.1.11.2 Oxidation degradation

25 mg of amlodipine besylate was weighed and transferred into 100 ml-volumetric flask, dissolved in10 ml of methanol and sonicated for 10 min. 10 ml of 3% hydrogen peroxide was added, and then the solution was heated on water bath at 80° C for 1 hour, cooled to room temperature and diluted to the mark with the diluent. The solution was filtered by using 45 µm filter. 5 ml of this solution was diluted to 25 ml using the diluent, and then it was analyzed using HPLC.

2.3.3.1.11.3 Thermal degradation

25 mg of amlodipine besylate was weighed and transferred into 100 ml-volumetric flask, dissolved in 10 ml of methanol and sonicated for 10 min. The solution was heated on water bath at 80° C for 1 hour, cooled to room temperature and dilute to the mark with the diluent. The solution was filtered by using 45 µm filter. 5 ml of this solution was diluted to 25 ml using the diluent, and then it was analyzed using HPLC.

2.3.3.1.11.4 Photolytic degradation

12.5 mg of amlodipine besylate was weighed and transferred into 50 ml-volumetric flask, dissolved in 10 ml of methanol and sonicated for 10 min. The solution was exposed to UV light at 254 nm for 16 hours. The solution was diluted to the mark with the diluent. The solution was filtered using 45 µm filter. 5 ml of this solution was diluted to 25 ml using the diluent, and then analyzed using HPLC.

2.3.3.2 HPLC method for the assay of amlodipine besylate: method 2

2.3.3.2.1 Preparation of mobile phase

20 mM of Potassium dihydrogen phosphate was prepared by dissolving 2.722 g of potassium dihydrogen phosphate and diluting it to 1 L, filtered and degassed before used.

2.3.3.2.2 Preparation of standard solution

A stock solution of 200 µg/ml amlodipine besylate standard was prepared by dissolving 10 mg of the standard in mobile phase, and then volume was madeup to 50 ml with the mobile phase.

2.3.3.2.3 Preparation of sample solution

20 tablets of amlodipine besylate (10 mg/tablet) were weighed and finely powdered. A portion of the powder equivalent to 10 mg of the drug was dissolved in the mobile phase, sonicated for 20 min, filtered and made up to 100 ml to obtain a solution of 100 µg/ml.

2.3.3.2.4 Validation of method 2

All validation parameters were carried out according to the method described in validation of method 1 except those for robustness.

2.3.3.2.4.1 Robustness

Robustness of the method was checked by varying sequentially each factor affecting the method and, then, observing the change in response. The factors which were studied are shown in Table (2.4).

Table (2.4) Method robustness with various parameters.

Flow rate (ml/min)			Wavelength of detection (nm)		
Level	Used	Original	Level	Used	Original
+1	1.65	1.5	+1	214	212
0	1.5		0	212	
-1	1.35		-1	210	

2.3.3.2.5 Preparation of solutions for degradation study

All preparations and procedure were carried out according to the method described in section 2.3.3.1.11.

2.3.3.3 HPLC method for the assay of esomeprazole magnesium: method 1

2.3.3.3.1 Optimization of method 1

Factorial design approach was used for optimization of chromatographic conditions for assay of esomeprazole magnesium. It was 2^3 full factorial design consisting of three factors; each factor had two levels. The three factors which affected the separation were: percentage of organic modifier methanol(X_1), percentage of water (X_2), and flow rate (X_3). The levels of each factor are shown in Table (2.5).

Table (2.5) Levels of factors employed in factorial design.

Factor	Level	
	-1	+1
Methanol %	60	65
Water %	40	45
Flow rate (ml/min)	1	1.2

Eight runs were performed to determine the optimum conditions for the chromatographic separation. The matrices of the eight runs are shown in Table (2.6).

Table (2.6) Matrices of 2^3 factorial optimization of mobile phase conditions for esomeprazole HPLC method 1.

Exp. No.	Methanol % (X_1)	Water % (X_2)	Flow rate (ml/min) (X3)
1	60	40	1.0
2	65	40	1.0
3	60	45	1.0
4	65	45	1.0
5	60	40	1.2
6	65	40	1.2
7	60	45	1.2
8	65	45	1.2

2.3.3.3.2 Preparation of mobile phase

Mobile phase consisting of 60% methanol and 40% water was prepared, filtered and degassed before it was used.

2.3.3.3.3 Preparation of standard solution

A stock solution of 200 µg/ml esomeprazole magnesium standard was prepared by dissolving 10 mg of the standard in mobile phase, and then volume was madeup to 50 ml with the mobile phase.

2.3.3.3.4 Preparation of sample solution

10 tablets of esomeprazole (40 mg/tablet) were weighed and finely powdered. A portion of the powder equivalent to 10 mg of the drug was dissolved in methanol, sonicated for 20 min, filtered and madeup to 100 ml with methanol to give a solution of 100 µg/ml.

2.3.3.3.5 Selectivity

A placebo blank containing 10 mg of microcrystalline cellulose, starch maize, talcum powder, and magnesium stearate was prepared in 100 ml-volumetric flask using mobile phase, then diluted to obtain a concentration of 10 µg/ml.

2.3.3.3.6 System suitability

System suitability was checked using a solution of 50% concentration which was injected six times.

2.3.3.3.7 Linearity and range

Serial concentrations from the stock solution of esomeprazole standard were prepared by diluting 0.5, 1, 2, 3, 4, 6, and 8 ml to 20 ml using mobile phase.

2.3.3.3.8 Precision

Precision was assessed using three solutions prepared from the stock standard solution, and then the three solutions were analyzed three times on the same day and also at three different days in order to determine intra-day and inter-day precision, respectively.

2.3.3.3.9 Recovery (accuracy)

Recovery was performed in three levels. Three different concentrations of mixed standard and tablet sample solution were prepared to obtain known concentrations. The recovery was calculated from calibration curve.

2.3.3.3.10 Robustness

Robustness of the method was checked by varying the factors affecting the method, each one at a time, and then observing the change in response. The factors which were studied in this method are shown in Table (2.7).

Table (2.7) Method robustness with various parameters.

Mobile phase composition			Flow rate (ml/min)			Wavelength of detection (nm)		
Level	Used	Original	Level	Used	Original	Level	Used	Original
+1	61:39	60:40	+1	1.1	1.0	+1	304	302
0	60:40		0	1.0		0	302	
-1	59:41		-1	0.9		-1	300	

2.3.3.3.11 Preparation of solutions for degradation study

2.3.3.3.11.1 Acid base degradation

20 mg of esomeprazole magnesium was weighed and transferred into 100 ml-volumetric flask, dissolved with 10 ml of methanol and sonicated for 10 min. 10 ml of 1 M sodium hydroxide and 0.025 M hydrochloric acid were added separately. The alkaline solutions was heated on water bath at 80° C for 45 min, cooled to room temperature, while the

acidic solution was left to stand at room temperature for 10 min. Both solutions were neutralized to pH 7 using 0.01 M sodium hydroxide or 0.5 M hydrochloric acid, then madeup the volume to the mark with mobile phase. These solutions were filtered by using 45 µm filter. 10 ml of each solution was diluted to 25 ml using mobile phase, and then was analyzed using HPLC. The acidic- and alkaline-forced degradations were carried out in the dark in order to prevent the possible effect of light.

2.3.3.3.11.2 Oxidation degradation

20 mg of esomeprazole magnesium was weighed and transferred into 100 ml-volumetric flask, dissolved in 10 ml of methanol and sonicated for 10 min. 10 ml of 1% hydrogen peroxide was added, and then the solution was heated on water bath at 80° C for 45 min, cooled to room temperature and madeup to volume with mobile phase. The solution was filtered by using 45 µm filter. 10 ml of this solution was diluted to 25 ml using mobile phase and then was analyzed using HPLC.

2.3.3.3.11.3 Thermal degradation

20 mg of esomeprazole magnesium was weighed and transferred into 100 ml-volumetric flask, dissolved in 10 ml of methanol and sonicated for 10 min. The solution was heated on water bath at 80° C for 45 min, cooled to room temperature and madeup to volume with mobile phase. The solution was filtered by using 45 µm filter. 10 ml of this solution was diluted to 25 ml using mobile phase and then was analyzed using HPLC.

2.3.3.3.11.4 Photolytic degradation

10 mg of esomeprazole magnesium was weighed and transferred into 50 ml-volumetric flask, dissolved in 10 ml of methanol and sonicated for 10 min. The solution was exposed to UV light at 254 nm for 16 hours. The solution was madeup to volume with mobile phase. The solution was filtered by using 45 µm filter. 10 ml of this solution was diluted to 25 ml using mobile phase and then was analyzed using HPLC.

2.3.3.4 HPLC method for the assay of esomeprazole magnesium: method 2

2.3.3.4.1 Optimization of method 2

2^3 full factorial design consisting of three factors, each of them had two levels. The three factors which affected the separation were percentage of organic modifier, ethanol(X_1), percentage of water (X_2), and flow rate (X_3).

The levels of each factor are shown in Table (2.8).

Table (2.8) Levels of factors employed in factorial design.

Factor	Level	
	+1	-1
Ethanol %	60	55
Water %	50	45
Flow rate (ml/min)	1.5	1

Eight runs were performed to determine the optimum conditions for the chromatographic separation. The matrices of the eight runs are shown in Table (2.9).

Table (2.9) Matrices of 2^3 factorial optimization of mobile phase conditions for esomeprazole magnesium HPLC method 2.

Exp. No.	Ethanol % (X_1)	Water % (X_2)	Flow rate (ml/min) (X_3)
1	55	45	1.0
2	60	45	1.0
3	55	50	1.0
4	60	50	1.0
5	55	45	1.5
6	60	45	1.5
7	55	50	1.5
8	60	50	1.5

2.3.3.4.2 Preparation of mobile phase

Mobile phase consisting of 60% ethanol and 40% water was prepared, filtered and degassed before it was used.

2.3.3.4.3 Preparation of standard solution

A stock solution of 100 µg/ml esomeprazole magnesium standard was prepared by dissolving 10 mg of the standard in mobile phase, and then volume was madeup to 100 ml with the mobile phase.

2.3.3.4.4 Preparation of sample solution

10 tablets of esomeprazole (40 mg/tablet) were weighed and finely powdered. A portion of the powder equivalent to 10 mg of the drug was dissolved in mobile phase, sonicated for 20 min, filtered and madeup to 100 ml with mobile phase to give a solution of 100 µg/ml.

2.3.3.4.5 Validation of method 2

All validation parameters were carried out according to the method described in validation of method 1.

2.3.3.4.6 Preparation of solutions for degradation study

All preparations and procedure were carried out according to the method described in section 2.3.3.3.11.

2.3.4 Kinetic stability of amlodipine besylate and esomeprazole magnesium

2.3.4.1 Effect of sun light on stability of both drugs solution

25 mg of each drug was weighed and transferred separately into 100 ml-volumetric flask, dissolved and madeup to mark with methanol for each drug. Both drug solutions were exposed directly to sun light. Aliquots were withdrawn at different-time intervals: 1, 2, 3, 4, 5 and 6 hour for amlodipine and at 10, 20, 30, 40, 50 and 60 min for esomeprazole. These were filtered by using 0.45 µm filter and then injected into HPLC system.

2.3.4.2 Thermal degradation of both drugs solution at 80° C

25 mg of each drug was weighed and transferred separately into 100 ml-volumetric flask, dissolved and madeup to mark with methanol. Both drug solutions were heated on water bath at 80℃. Aliquots were withdrawn at 1, 2, 3, 4, 5 and 6 hours for both drugs. These were filtered by using 0.45 µm filter and then injected into HPLC system.

2.3.4.3 Effect of excepients on the thermal stability of both drugs solution

25 mg of each drug and 0.01 mg of each excipient were weighed and transferred separately into 100 ml-volumetric flask, dissolved and madeup to mark with methanol for each one. These solutions were heated on water bath at 80℃. Aliquots were withdrawn at 1, 2, 3, 4, 5 and 6 hours, for both drugs. Aliquots were filtered by using 0.45 µm filter and then injected into HPLC system.

2.3.4.4 Effect of controlled temperature 100° C on stability of both solid drugs

A few grams of each drug was placed in thermostatic vacuum oven at 100℃; portions of the sample were withdrawn every day for five days. These samples were treated to obtain a concentration of 30 µg/ml and then analyzed by HPLC method 1 for each drug.

2.3.4.5 Thermal degradation of amlodipine besylate solution in alkaline medium

25 mg of amlodipine besylate was weighed and transferred into 100 ml-volumetric flask, dissolved in 10 ml of methanol and sonicated for 10 min. 10 ml of 1 M sodium hydroxide was added and the volume was madeup to mark with methanol. The solution was heated on thermostatic water bath at 80℃. Aliquots were withdrawn at 20, 40, 60, 80, 100 and 120 min. These aliquots were filtered by using 0.45 µm filter and then injected into HPLC system.

2.3.5.4 Acid degradation of esomeprazole magnesium solution at room temperature

25 mg of esomeprazole magnesium was weighed and transferred into 100 ml-volumetric flask, dissolved in 10 ml of methanol and sonicated for 10 min. 10 ml of 0.01 M hydrochloric acid was added and madeup to mark with methanol. The solution was left at room temperature. Aliquots were withdrawn at 5, 15, 25, 35, 45 and 55 min. These aliquots were filtered by using 0.45 µm filter and then injected into HPLC system.

2.3.5.5 Peroxide degradation of esomeprazole magnesium solution at room temperature

25 mg of esomeprazole magnesium was weighed and transferred into 100 ml-volumetric flask, dissolved in 10 ml of methanol and sonicated for 10 min. 10 ml of 3% hydrogen peroxide was added, and madeup to mark with methanol. The solution was left at room temperature. Aliquots were withdrawn at 10, 20, 30, 40, 50 and 60 min. These aliquots were filtered by using 0.45 µm filter and then injected into HPLC system.

2.3.5.6 Photo stability of esomeprazole magnesium solution at 254 nm

12.5 mg of esomeprazole magnesium was weighed and transferred into 50 ml-volumetric flask, dissolved in 10 ml of methanol and sonicated for 10 min and madeup to mark with methanol. The solution was exposed to UV light at 254 nm. Aliquots were withdrawn at 1, 2, 3, 4, and 6 hours. These aliquots were filtered by using 0.45 µm filter and then injected into HPLC system.

3 RESULTS AND DISSCISSION

3.1 Identification

3. 1.1 I.R identification test for amlodipine besylate

The IR spectrum of amlodipine besylate shows characteristic peaks corresponding to C=O, C-O, S=O, N-H, S-O, C-N, S-O and C=C (aromatic) as shown in Table (3.1), Figure (3.1).

Table (3.1) IR frequencies of amlodipine besylate.

Functional group	Band frequency (cm^{-1})
C=O	1698.02 and 1674.87
C-O	1302.68 and 1125.26
C=C (aromatic)	1493.6 and 1469.49
S=O	1206.26 "asym" and 1093.44 "sym"
S-O	615.181
N-H	3444.24 "asym" and 3164.61 "sym"
C-N	1264.11

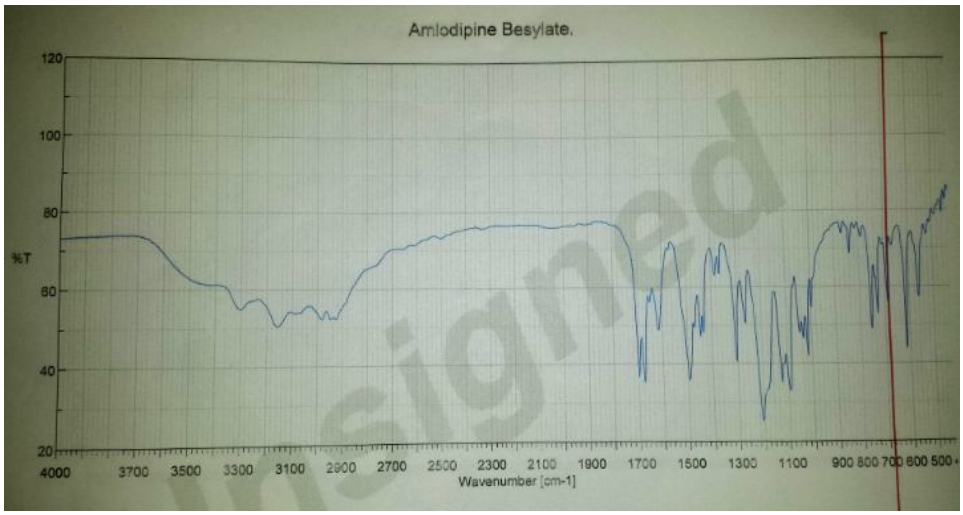

Figure (3.1) IR spectrum of amlodipine besylate standard.

3.1.2. Effect of solvents on the UV spectrum of amlodipine besylate

An absorbance characteristic of amlodipine in different solvents was studied in order to determine the suitable solvent to dissolve the drug. Amlodipine besylate showed almost the same absorbance bands in 2-propanol, acetonitrile, and methanol. In ethanol it showed slightly different behavior. Absorbance of amlodipine in different solvents was in the following order:

2-propanol > methanol > ethanol > acetonitrile, as shown in Figure (3.2).

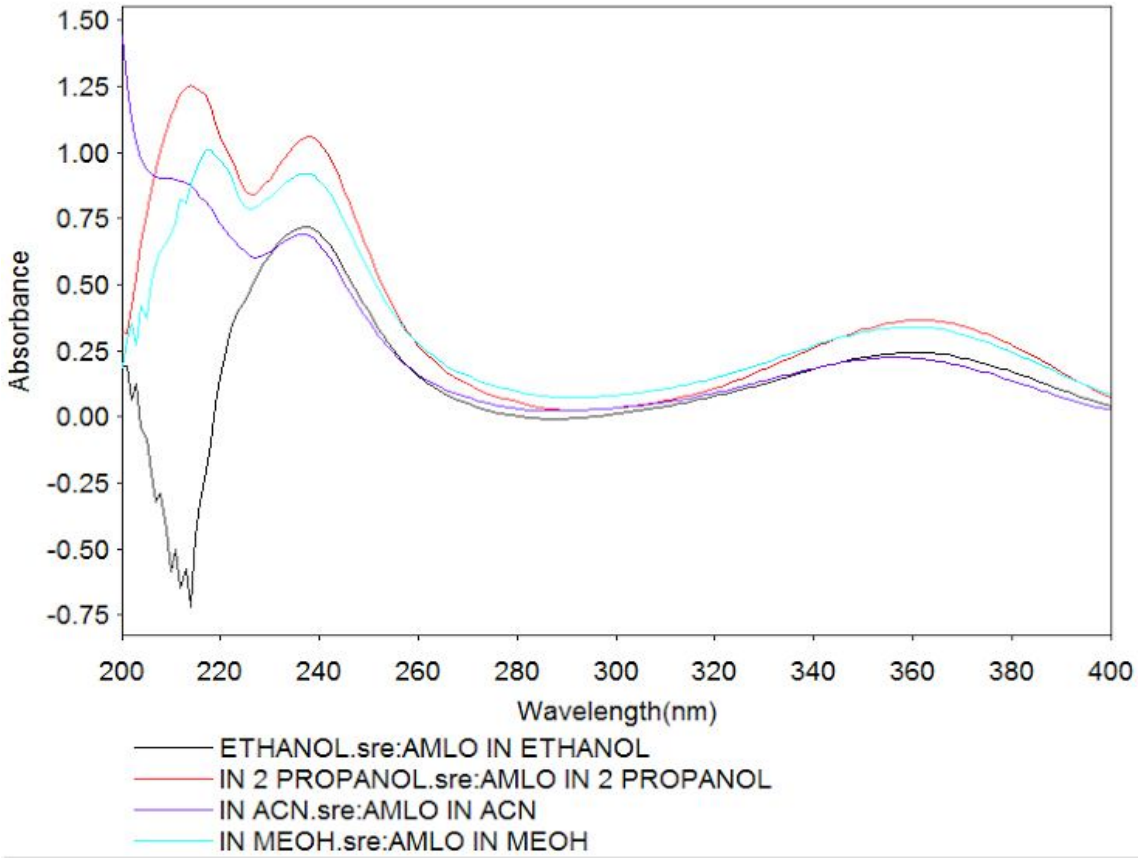

Figure (3.2) Effect of solvent on UV spectrum of amlodipine besylate.

3.1.3 I.R identification test for esomeprazole magnesium

The IR spectrum of amlodipine besylate shows characteristic peaks corresponding to C-O, C=N, N-H, S=O, C-N, C=C, C-H, N=H, and C=C (aromatic) as shown in Table (3.2), Figure (3.3).

Table (3.2) IR frequencies of esomeprazole magnesium.

Functional group	Band frequency (cm^{-1})
C-O (with pyridine ring)	1198.54 and 1013.41
C-O (with benzene ring)	1272 and 1029.8
C=N	1612.2
C-N	1297.26
C=C aromatic	1571 and 1477.21
S=O	1077.05
C-H	3000 "asym" and 2930 "sym"
N-H	3420

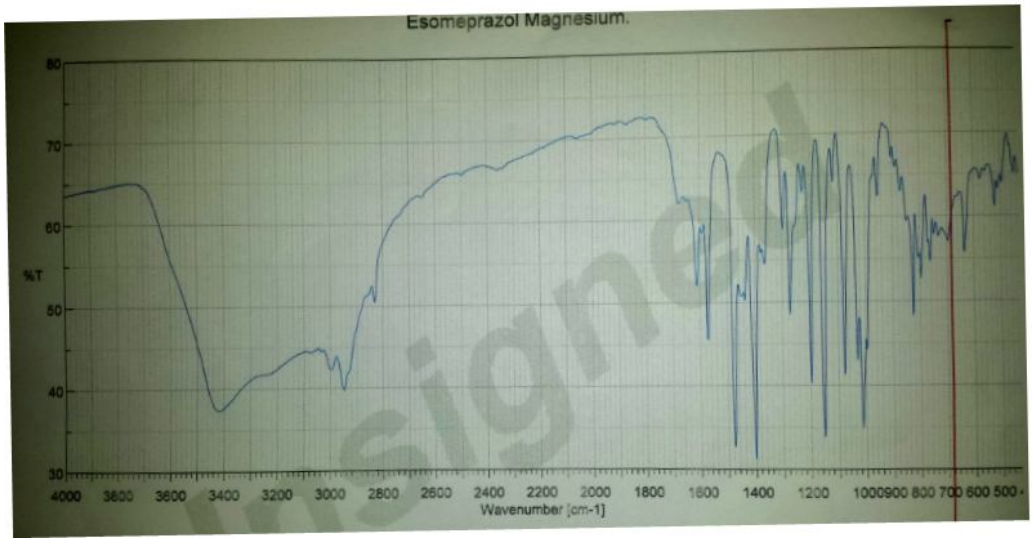

Figure (3.3) IR spectrum of esomeprazole magnesium standard.

3.1.4. Effect of solvents on the UV spectrum of esomeprazole magnesium

Esomeprazole in different solvents showed almost the same absorbance characteristics; absorbance was in the following order:

2-propanol > acetonitrile > ethanol > acetone > methanol, as shown in Figure (3.4).

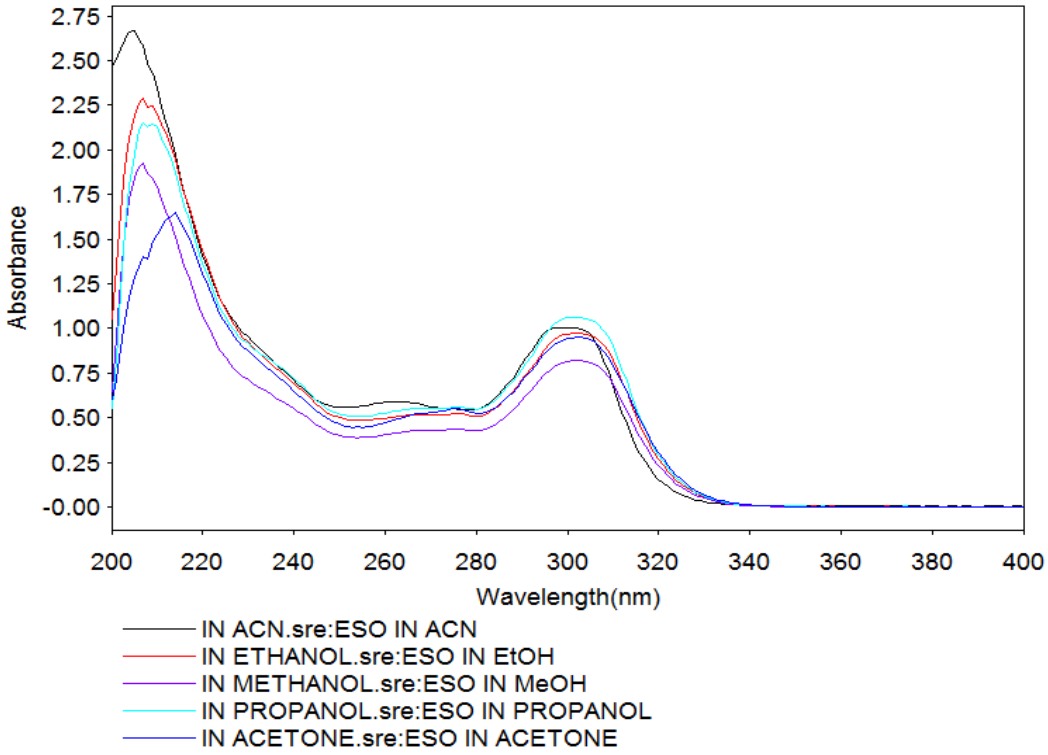

Figure (3.4) Effect of solvent on UV spectrum of esomeprazole magnesium.

3.2 UV- visible Method development and validation

3.2.1 Development and validation of spectrophotometric methods for determination of amlodipine besylate

3.2.1.1 Method 1

3.2.1.1.1 Selection of solvent

Solubility of amlodipine besylate was studied using different hydrotropic agents including 0.5 M potassium acetate (a), 0.5 M ammonium acetate (b), 0.5 M nicotineamide (c), 1 M sodium citrate, 1 M ascorbic acid and 1 M sodium salicylate. Solubility of amlodipine besylate was found to increase when using potassium acetate, ammonium acetate, and nicotineamide.

3.2.1.1.2 Determination of λ_{max}

Absorption spectrum of amlodipine in ammonium acetate, potassium acetate and nicotineamide was recorded in the range 200-400 nm as shown in Figure (3.5). It shows that the maximum absorption wavelength (λ_{max}) is at 365 nm for the three hydrotropic agents.

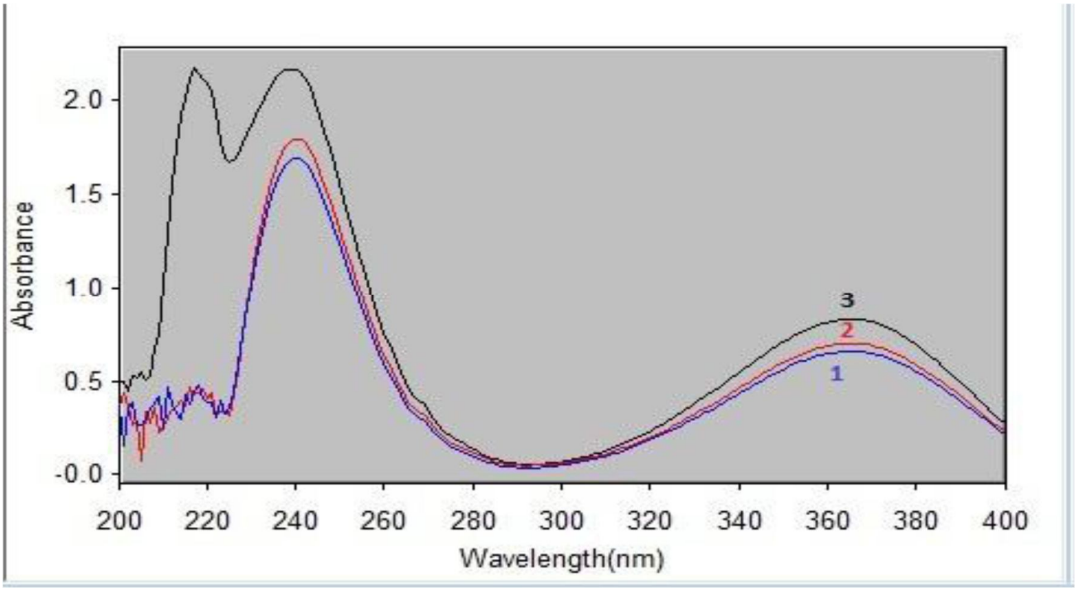

Figure (3.5): Absorption spectra of amlodipine in (1) potassium acetate, (2) ammonium acetate and (3) nicotinamide.

3.2.1.1.3 Linearity and range

The linearity of the methods were investigated and found to be in the range of 5- 90 µg/ml for the three methods as shown in Figure (3.6).

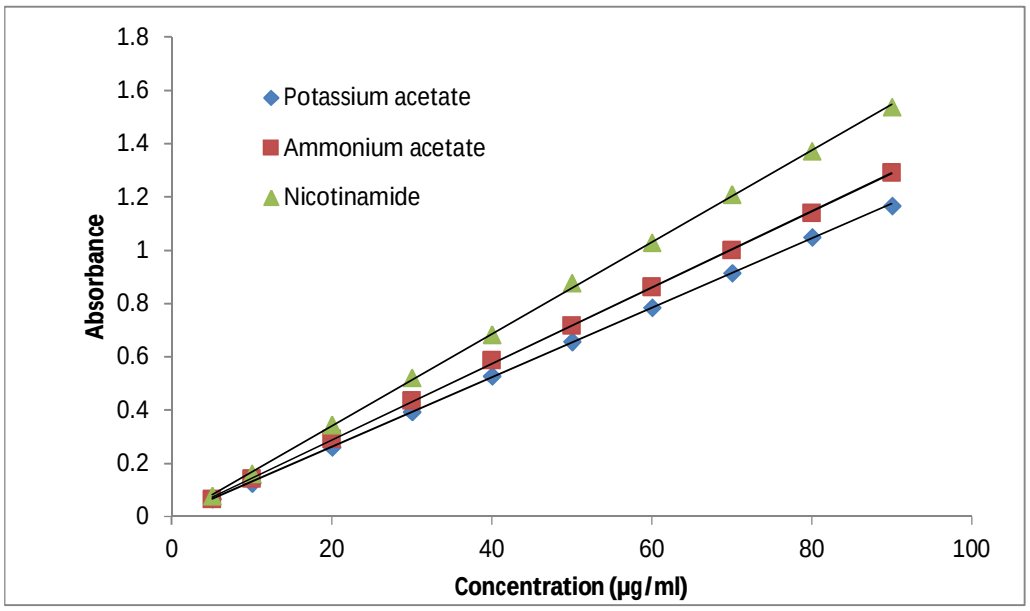

Figure (3.6) Calibration curves for determination of amlodipine using hydrotropic agents.

3.2.1.1.4 Precision

To assess the precision, each experiment was repeated three times on the same day (intra-day) and at different days (inter-day). The results showed that the methods were precise according to the low values of standard deviation (SD) and percent relative standard deviation (% RSD), as shown in Table (3.3).

Table (3.3) Evaluation of intra- and inter-day precision of the proposed spectrophotometric method 1 for assay of amlodipine besylate.

Method	Taken (µg/ml)	Intra – day precision			Inter – day precision		
		Found (µg/ml)	SD	% RSD	Found (µg/ml)	SD	% RSD
Method a	10	10.098	0.058	0.476	10.190	0.049	0.476
	20	20.179	0.034	0.166	20.217	0.051	0.253
	30	30.008	0.058	0.193	30.050	0.039	0.130
Method b	10	9.975	0.044	0.444	9.941	0.039	0.393
	20	20.047	0.044	0.221	20.029	0.053	0.266
	30	30.272	0.077	0.253	30.186	0.078	0.259
Method c	10	9.988	0.0404	0.404	9.957	0.036	0.358
	20	20.104	0.040	0.201	20.088	0.0931	0.464
	30	30.361	0.001	0.464	30.322	0.036	0.117

3.2.1.1.5 Accuracy (Recovery Studies)

Accuracy was estimated in terms of percent recovery and percent relative standard deviation. t-test and F-test values had been calculated using USP standard reference HPLC method. These were less than their permissible values of 2.776, and 19.0 for t- and F-test, respectively, at 95% confidence level, indicating a high accuracy and precision of the methods as shown in Table (3.4).

Table (3.4) Accuracy studies of the proposed spectrophotometric method 1 for assay of amlodipine besylate.

Method	Taken (µg/ml)	Found (µg/ml)	% Recovery	% RSD	Proposed Method Mean ± SD	Reference Method Mean ± SD	t-test	F-test
Method a	5	5.009	100.172	0.263	100.04 ± 0.26	99.77 ± 0.30	1.155	1.316
	10	10.020	100.201					
	20	19.946	99.732					
Method b	5	5.016	99.558	0.263	99.74 ± 0.26		0.114	1.319
	10	9.975	99.626					
	20	20.046	100.043					
Method c	5	4.9995	99.991	0.161	99.83 ± 0.16		0.315	4.317
	10	9.965	99.879					
	20	19.988	99.939					

3.2.1.1.6 Limit of detection and limit of Quantitation

Limit of detection (LOD) and limit of quantification (LOQ) were determined from the regression equation of calibration curves.

For method a: LOD = 1.072 µg/ml, LOQ = 3.249 µg/ml.

For method b: LOD = 0.646 µg/ml, LOQ = 1.956 µg/ml.

For method c: LOD = 0.866 µg/ml, LOQ = 2.625 µg/ml.

3.2.1.1.7 Robustness

Robustness was examined by evaluating the influence of small variation in the method variables on its analytical performance. In these experiments, one parameter was changed, whereas the others were kept unchanged, and the recovery percentage was calculated each time. It was found that small variation in the method variables did not significantly affect the procedure's recovery values, as shown in Table (3.5).

Table (3.5) Robustness of the proposed spectrophotometric method 1 for assay of amlodipine besylate.

Recommended condition		Recovery (Mean ± SD)*		
		Method a	**Method b**	**Method c**
Standard		100.04 ± 0.263	99.74 ± 0.2624	101.14 ± 0.217
λ_{max}	363	100.01 ± 0.341	99.64 ± 0.444	100.28 ± 0.106
	367	99.81 ± 0.582	100.02 ± 0.221	100.23 ± 0.200
Concentration of hydrotropic agent	0.4 M	99.91 ± 0.444	99.97 ± 0.277	99.97 ± 0.241
	0.6 M	100.11 ± 0.290	100.02 ± 0.221	100.02 ± 0.223

***** Values are mean of three determinations.

All analytical parameters for spectrophotometric determination of amlodipine besylate using method 1 were summarized in Table (3.6).

Table (3.6) Analytical parameters for the proposed spectrophotometric 1 for the determination of amlodipine besylate.

Parameter	Value		
	Method a	**Method b**	**Method c**
λ_{max} (nm)	365	365	365
Beer's law limits (µg/ml)	5 - 90	5 - 90	5-90
Sandell's sensitivity (μgcm^{-2})	0.0581	0.0767	0.0699
Molar absorptivity ($lmol^{-1}cm^{-1}$)	0.977×10^4	0.740×10^4	0.812×10^4
Std. Dev. of intercept	0.005598	0.002551	0.003759
LOD (µg/ml)	1.072	0.646	0.866
LOQ (µg/ml)	3.249	1.956	2.625
Slope (m)	0.0172	0.01304	0.0143
Intercept (b)	- 0.0003	0.00074	0.0084
Correlation coefficient	0.9997	0.9999	0.9998
Regression equation	y = 0.0172x - 0.0003	y = 0.01304x + 0.00074	y = 0.0143x + 0.0084

3.2.1.2 Method 2

3.2.1.2.1 Selection of solvent

Methanol and water was chosen as a solvent for amlodipine besylate, and water was used as a solvent for methyl orange.

3.2.1.2.2 Determination of λ_{max}

Absorption spectrum of the product formed from the reaction between amlodipine and methyl orange at pH 3.5 was scanned in the range 250-600 nm as in Figure (3.7). It shows that the maximum absorption wavelength (λ_{max}) was at 427 nm.

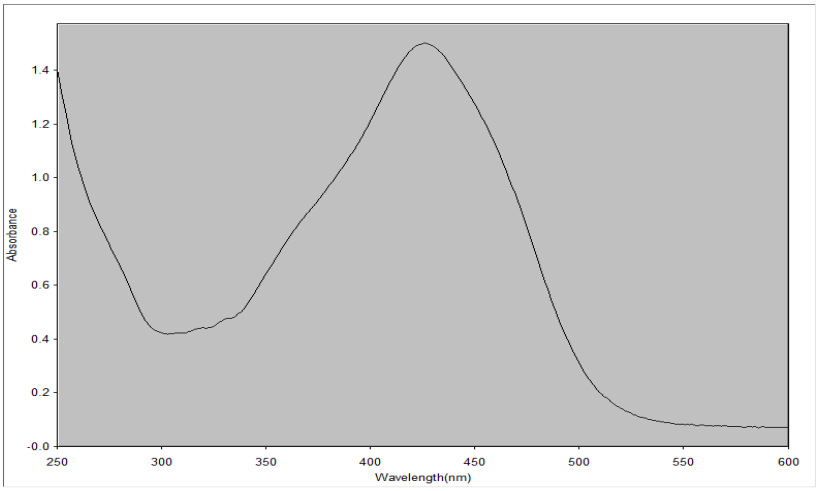

Figure (3.7) Absorption spectra of amlodipine (10 µg/ml) with M.O. at pH 3.5 against blank.

3.2.1.2.3 Optimization of reaction conditions

3.2.1.2.3.1 Effect of volume of reagent

The effect of volume of methyl orange was studied in the range from 0.5 to 3 ml. Optimum result was obtained with 2 ml of methyl orange since it gave the maximum absorbance as in Figure (3.8).

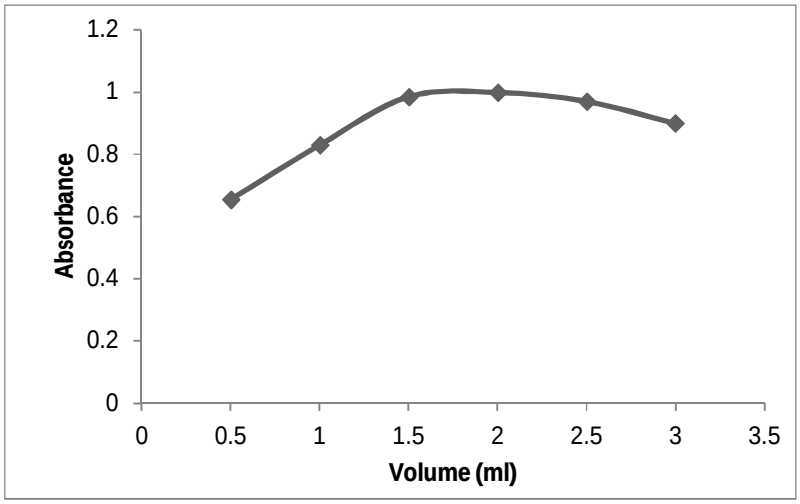

Figure (3.8) Effect of volume of reagent on the reaction between amlodipine and M.O.

3.2.1.2.3.2 Effect of pH

The effect of pH of the buffer solution was studied in the range of 2 to 4.5. Optimum pH was found to be 3.5 as shown in Figure (3.9).

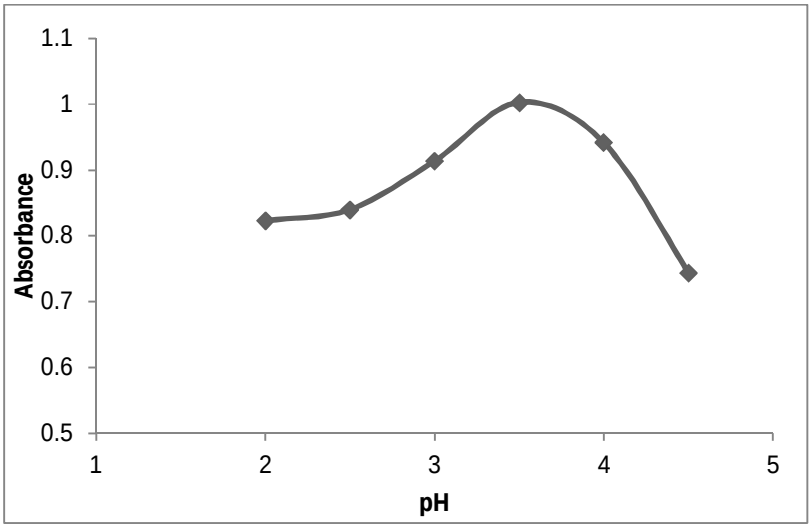

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Figure (3.9) Effect of pH on the reaction between amlodipine and M.O.

3.2.1.2.3.3 Effect of volume of buffer

The effect of volume of buffer was studied in the range of 0.25 to 2 ml. Optimum volume was found to be 1 ml as in Figure (3.10).

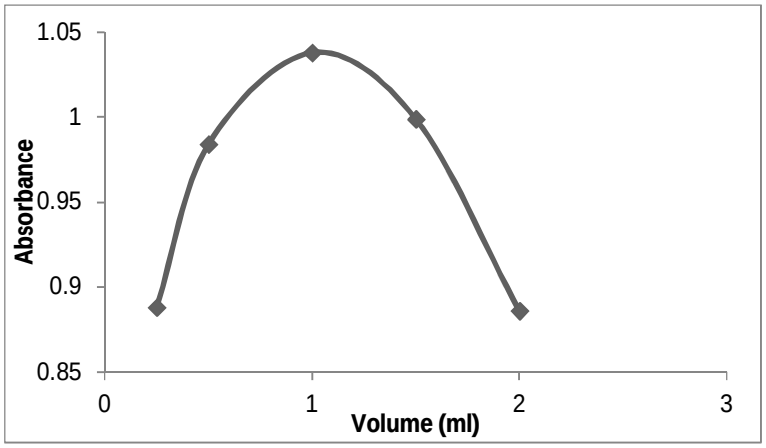


Figure (3.10) Effect of volume of buffer on the reaction between amlodipine and M.O.

All conditions affecting the reaction were optimized and summarized in Table (3.7).

Table (3.7) Optimum conditions for reaction between amlodipine and methyl orange.

Condition	**Optimum**
Reagent concentration % (w/v)	0.01
Volume of reagent (ml)	2.0
pH	3.5
Volume of buffer (ml)	10
Reaction time (min)	Immediately

3.2.1.2.4 Determination of stoichiometry of the reaction

Under optimum conditions, as shown in Table (3.6), the stoichiometry of the reaction between amlodipine and methyl orange was investigated by Job's method and was found to be 1:1, as shown in Figure (3.11). The reaction pathway was postulated to proceed as shown in Scheme (3.1).

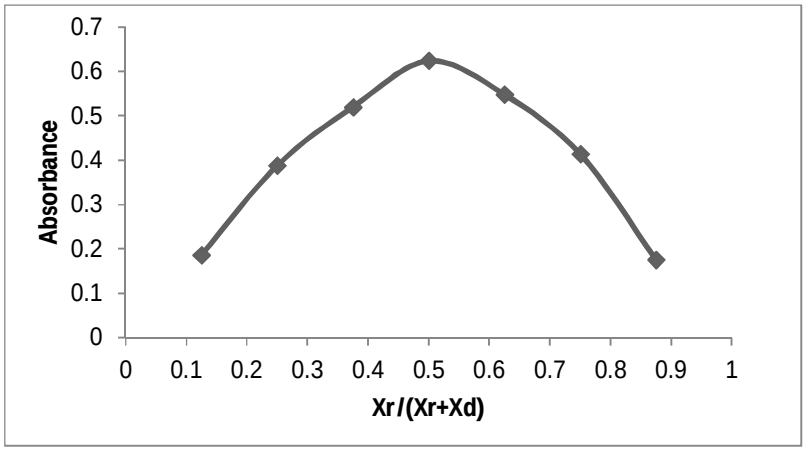
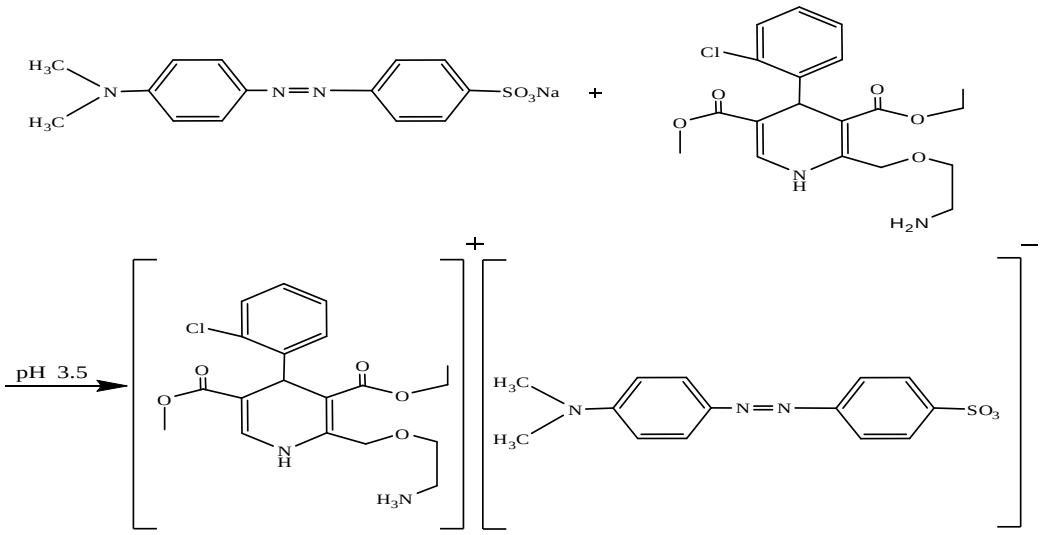


Figure (3.11) Continuous-variation plot for stoichiometry of the reaction between amlodipine and methyl orange.



Scheme (3.1) Reaction pathway of amlodipine with M.O.

3.2.1.2.5 Linearity and range

The linearity of the method was investigated and found to be in the range of 2.5 - 30 µg/ml, as shown in Figure (3.12).

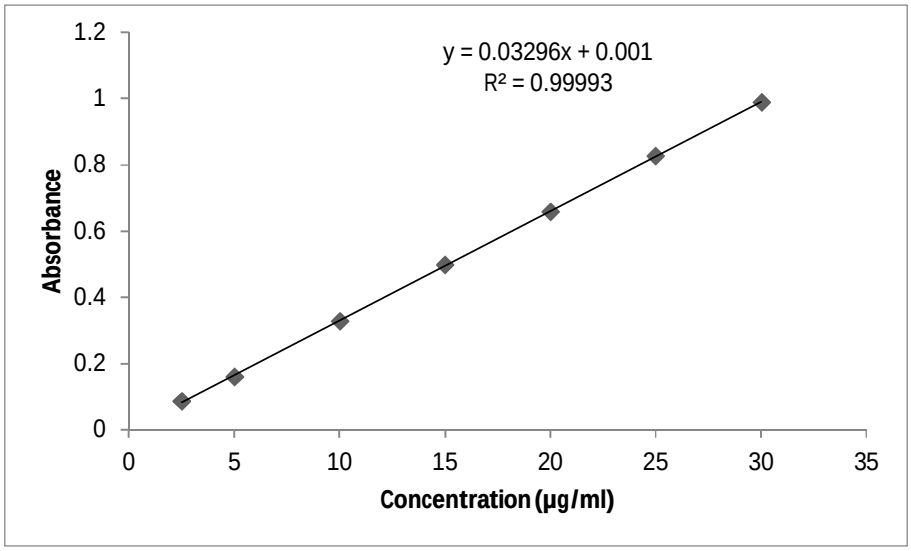


Figure (3.12) Calibration curve for determination of amlodipine using methyl orange.

3.2.1.2.6 Precision

To assess the precision, each experiment was repeated three times on the same day (intra-day), and at different days (inter-day). The results showed that the method was precise according to the low values of standard deviation (SD), and percent relative standard deviation (% RSD), as shown in Table (3.8).

Table (3.8) Evaluation of intra- and inter-day precision of the proposed spectrophotometric method 2 for assay of amlodipine besylate.

Taken (µg/ml)	Intra – day precision			Inter – day precision		
	Found (µg/ml)	SD	% RSD	Found (µg/ml)	SD	% RSD
6	6.14	0.045	0.730	6.142	0.015	0.250
8	8.18	0.045	0.550	8.186	0.025	0.310
12	11.91	0.013	0.110	11.913	0.0153	0.130

3.2.1.2.7 Recovery Studies (Accuracy)

Accuracy was estimated in terms of percent recovery and percent relative standard deviation. t-test and F-test values had been calculated using USP standard reference HPLC method. The t-test and F-test values were less than their permissible values: 2.776 and 19.0 for t- and F-test, respectively, at 95% confidence level, indicating a high accuracy and precision of the method as shown in Table (3.9).

Table (3.9) Accuracy studies of the proposed spectrophotometric method 2 for assay of amlodipine besylate.

Taken (µg/ml)	**Found (µg/ml)**	**% Recovery**	**% RSD**	**Proposed Method Mean ± SD**	**Reference Method Mean ± SD**	**t-test**	**F-test**
10	10.08	100.79	0.644	100.56 ± 0.65	99.77 ± 0.30	1.35	1.38
15	15.16	101.05					
20	19.96	99.82					

3.2.1.2.8 Limit of detection and limit of Quantitation

Limit of detection (LOD) and limit of quantification (LOQ) were determined from the regression equation of calibration curves.

LOD = 0.224 µg/ml, LOQ = 0.80 µg/ml.

3.2.1.2.9 Robustness

Robustness was examined by evaluating the influence of small variation in the method variables on its analytical performance. In these experiments, one parameter was changed whereas the others were kept unchanged, and the recovery percentage was calculated each time. It was found that small variation in the method variables did not significantly affect the procedure's recovery values, as shown in Table (3.10).

Table (3.10) Robustness of the proposed spectrophotometric method 2 for assay of amlodipine besylate.

Recommended condition	**Recovery (Mean ± SD)***	
Standard	100.56 ± 0.65	
Reagent concentration 0.01%(w/v)	0.005	99.91 ± 0.38
	0.015	100.29 ± 0.28
λ_{max} (427 nm)	425	99.4 ± 0.22
	429	98.65 ± 0.22
pH (3.5)	3.3	100.06 ± 0.3
	3.7	99.40 ± 0.22

***** Values are mean of three determinations

All analytical parameters for spectrophotometric determination of amlodipine besylate using method 2 were summarized in Table (3.11).

Table (3.11) Analytical Parameters for the proposed spectrophotometric 2 for the determination of amlodipine besylate.

Parameter	Value
λ_{max} (nm)	427
Beer's law limits (µg/ml)	2.5 - 30
Sandell's sensitivity (µgcm^{-2})	0.03030
Molar absorptivity (lmol^{-1}cm^{-1})	1.871×10^{4}
Std. Dev. of intercept	0.002244
LOD (µg/ml)	0.224
LOQ (µg/ml)	0.800
Slope (m)	0.03296
Intercept (b)	0.001
Correlation coefficient	0.99993
Regression equation	y = 0.03296x + 0.001

3.2.2 Development and validation of spectrophotometric methods for determination of esomeprazole magnesium

3.2.2.1 Method 1

3.2.2.1.1 Selection of solvent

Methanol was chosen as a solvent for esomeprazole magnesium and alizarin red s.

3.2.2.1.2 Determination of λ_{max}

Absorption spectrum of the product formed from the reaction between esomeprazole and alizarin red s was recorded in the range 300-600 nm (Figure 3.13). The maximum absorption wavelength (λ_{max}) was at 521 nm.

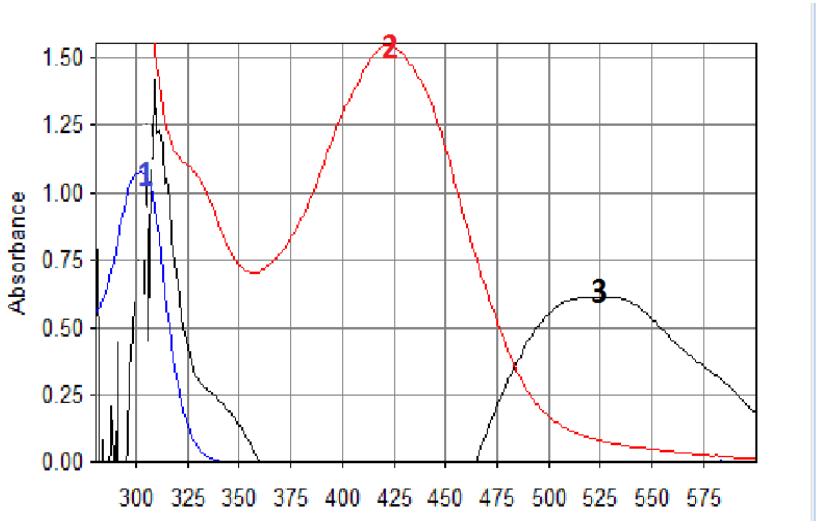


Figure (3.13) Absorption spectra of (1) esomeprazole against methanol blank, (2) Alizarin red S against methanol blank, (3) the reaction product against reagent blank.

3.2.2.1.3 Optimization of reaction conditions

3.2.2.1.3.1 Effect of volume of reagent

The effect of volume of alizarin red s was studied in the range of 0.5 to 3 ml. Optimum result was obtained with 2 ml of alizarin red s as in Figure (3.14).

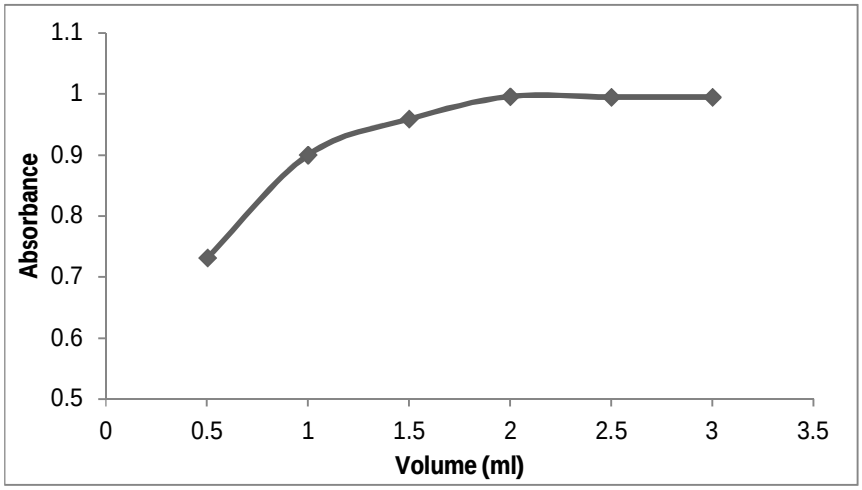


Figure (3.14) Effect of volume of 0.2 % alizarin on the reaction between esomeprazole and alizarin red s.

3.2.2.1.3.2 Effect of temperature

The influence of temperature was studied in the range of 30°C to 50 °C. The optimum temperature was found to be 40 °C as shown in Figure (3.15).

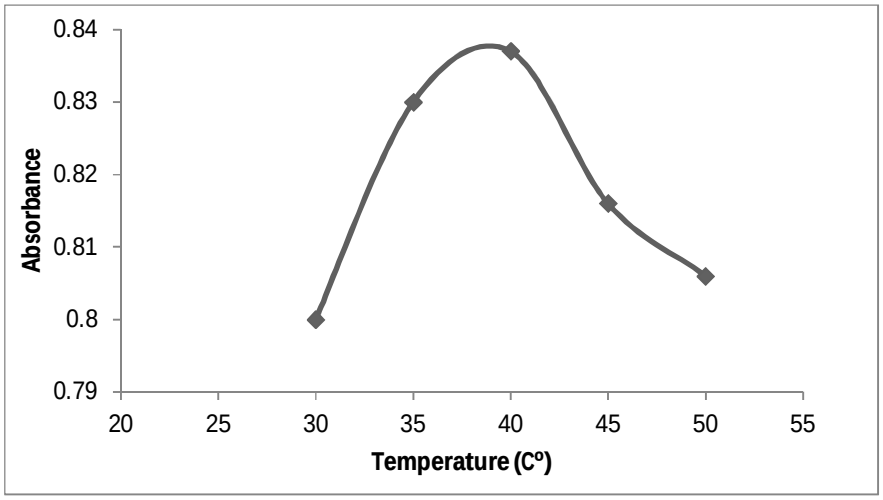


Figure (3.15) Effect of temperature on the reaction between esomeprazole and alizarin red s.

3.2.2.1.3.3 Effect of heating time

In order to obtain the highest and stable absorbance, the effect of heating time was checked out on a water bath at 40 ± 1 °C for periods ranging between 5 to 25 min. The optimum time required to complete the reaction was found to be 15 min of heating as in Figure (3.16).

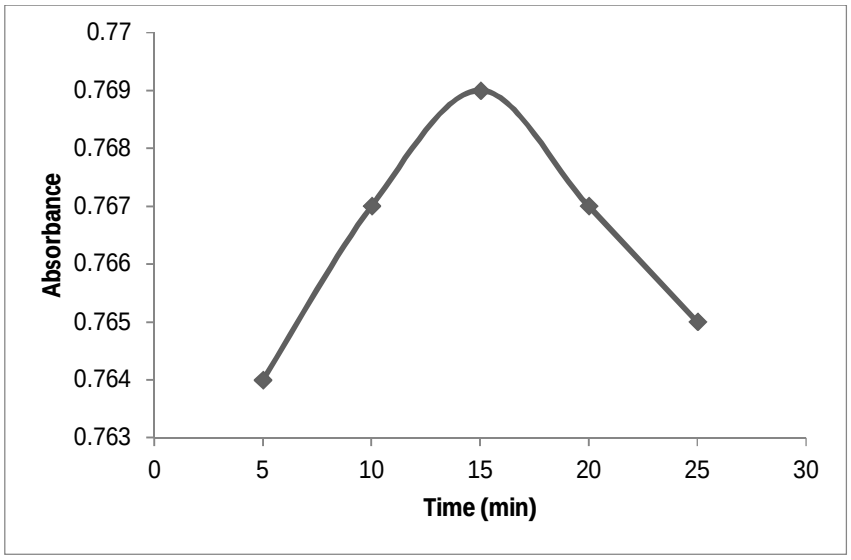


Figure (3.16) Effect of time of heating on the reaction between esomeprazole and alizarin red s.

All conditions affecting the reaction were optimized and summarized in Table (3.12).

Table (3.12) Optimum conditions for reaction between esomeprazole and alizarin red s.

Condition	**Optimum**
Reagent concentration % (w/v)	0.2
Volume of reagent (ml)	2.0
Temperature (℃)	40
Reaction time (min)	15

3.2.2.1.3 Determination of stoichiometry of the reaction

Under optimum conditions, as shown in Table (3.10), the stoichiometry of the reaction between esomeprazole and alizarin was investigated by Job's method and was found to be 1:1, as shown in Figure (3.17). The reaction pathway was postulated to proceed as shown in Scheme (3.2).

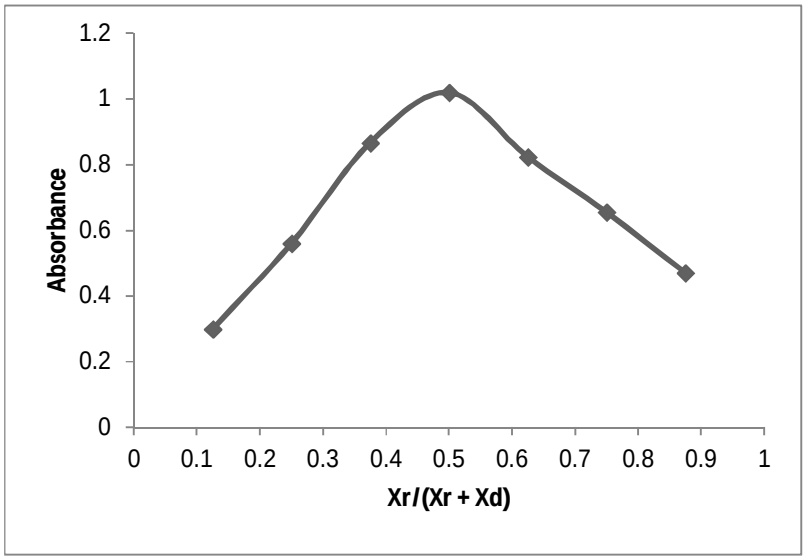
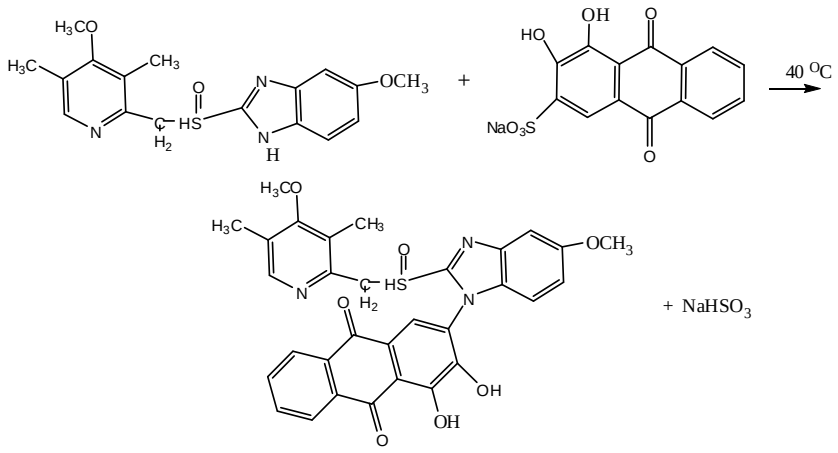


Figure (3.17) Continuous variation plot for stoichiometry of the reaction between esomeprazole and alizarin red s.



Scheme (3.2) Reaction pathway of esomeprazole with alizarin.

3.2.2.1.4 Linearity and range

The linearity of the method was investigated and found to be in the range of 5 - 80 µg/ml, as shown in Figure (3.18).

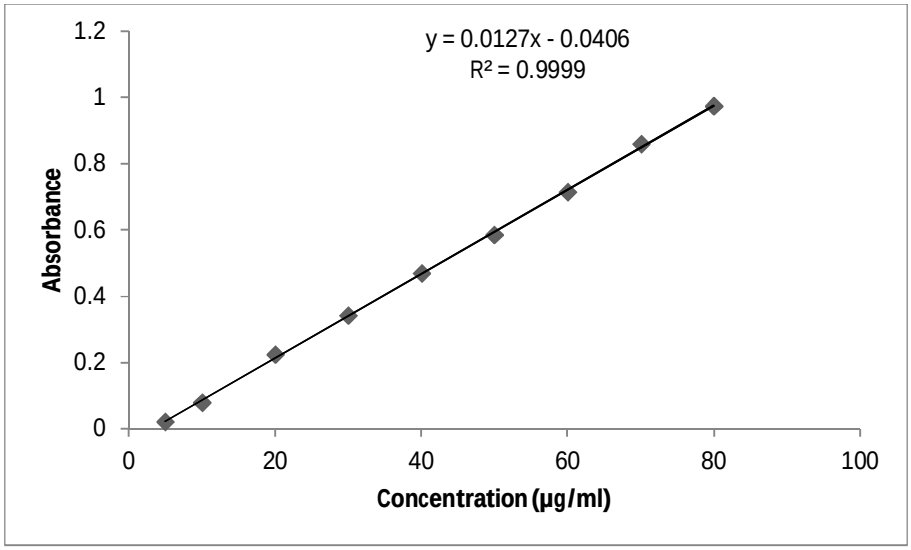


Figure (3.18) Calibration curve for determination of esomeprazole using alizarin red s.

3.2.2.1.5 Precision

To assess the precision, each experiment was repeated three times on the same day (intra-day), and at different days (inter-day). The results showed that the method was precise according to the low values of standard deviation (SD), and percent relative standard deviation (% RSD), as shown in Table (3.13).

Table (3.13) Evaluation of intra- and inter-day precision of the proposed spectrophotometric method 1 for assay of esomeprazole magnesium.

Taken (µg/ml)	Intra – day precision			Inter – day precision		
	Found (µg/ml)	SD	% RSD	Found (µg/ml)	SD	% RSD
15	14.93	0.04	0.27	15.14	0.065	0.43
25	24.93	0.075	0.30	24.85	0.051	0.21
35	34.87	0.12	0.33	35.16	0.078	0.22

3.2.2.1.6 Accuracy (Recovery Studies)

Accuracy was estimated in terms of percent recovery and percent relative standard deviation. t-test and F-test values had been calculated using USP standard reference HPLC method. These values were less than their permissible values of 2.776, and 19.0 for t- and F-test, respectively, at 95% confidence level, indicating a high accuracy and precision of the method as shown in Table (3.14).

Table (3.14) Accuracy studies of the proposed spectrophotometric method 1 for assay of esomeprazole magnesium.

Taken (µg/ml)	Found (µg/ml)	% Recovery	% RSD	Proposed Method Mean ± SD	Reference Method Mean ± SD	t-test	F-test
30	29.89	99.63	0.277	99.74 ± 0.276	99.97 ± 0.416	0.798	2.272
40	39.81	99.53					
50	49.73	100.05					

3.2.2.1.7 Limit of detection and limit of Quantitation

Limit of detection (LOD) and limit of quantification (LOQ) were determined from the regression equation of calibration curves.

LOD = 1.69 µg/ml, LOQ = 5.37 µg/ml.

3.2.2.1.8 Robustness

Robustness was examined by evaluating the influence of small variation in the method variables on its analytical performance. In these experiments, one parameter was

changed whereas the others were kept unchanged, and the recovery percentage was calculated each time. It was found that small variation in the method variables did not significantly affect the procedure's recovery values, as shown in Table (3.15).

Table (3.15) Robustness of the proposed spectrophotometric method 1 for assay of esomeprazole magnesium.

Recommended condition	**Recovery (Mean ± SD)***	
Standard	99.74 ± 0.276	
Reagent concentration 0.2%(w/v)	0.15	99.11 ± 0.001
	0.25	98.77 ± 0.001
λ_{max} (521 nm)	519	99.58 ± 0.001
	523	100.08 ± 0.0006
Heating time (15 min)	13	100.24 ± 0.0015
	17	99.03 ± 0.0006

***** Values are mean of three determinations

3.2.2.2 Method 2

3.2.2.2.1 Selection of solvent

Methanol and water were used as solvents for both esomeprazole and eriochrome black T. Chloroform was used in the extraction process.

3.2.2.2.2 Determination of λ_{max}

Absorption spectrum of the product formed from the reaction between esomeprazole and EBT at pH 3 was recorded in the range 300-650 nm as shown in Figure (3.19). The maximum absorption wavelength (λ_{max}) was at 511 nm.

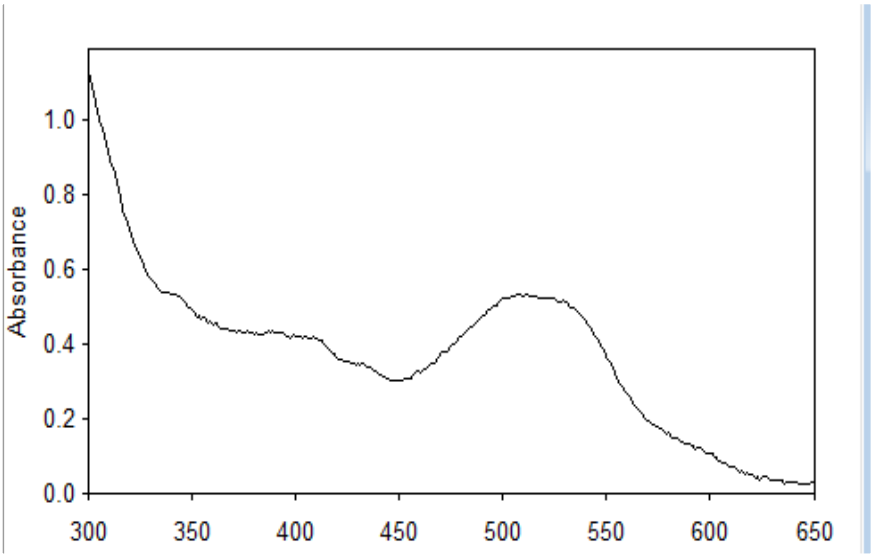


Figure (3.19) Absorption spectra of ESO (10 µg/ml) with EBT at pH 3 against blank.

3.2.2.2.3 Optimization of reaction conditions

3.2.2.2.3.1 Effect of volume of reagent

The effect of volume of EBT was studied in the range from 1 to 3 ml. Optimum result was obtained with 2 ml of EBT as shown in Figure (3.20).

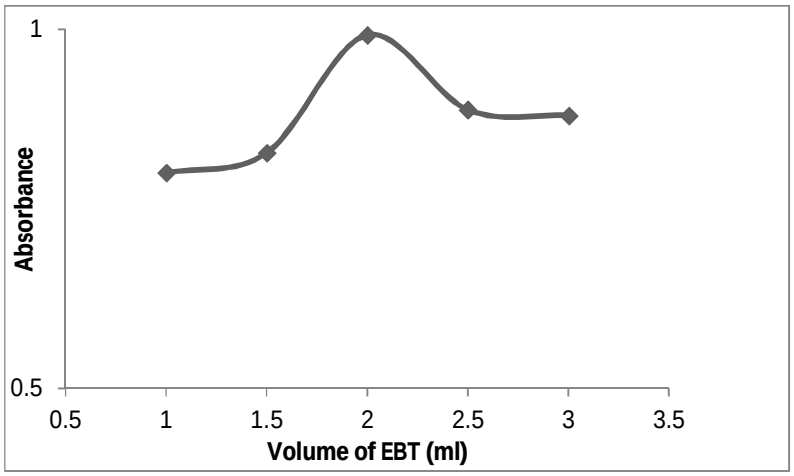


Figure (3.20) Effect of volume of 0.5 % EBT on the reaction between esomeprazole and EBT.

3.2.2.2.3.2 Effect of pH

The effect of pH of the buffer solution was studied in the range of 2 to 4. Optimum pH was found to be 3 as shown in Figure (3.21).

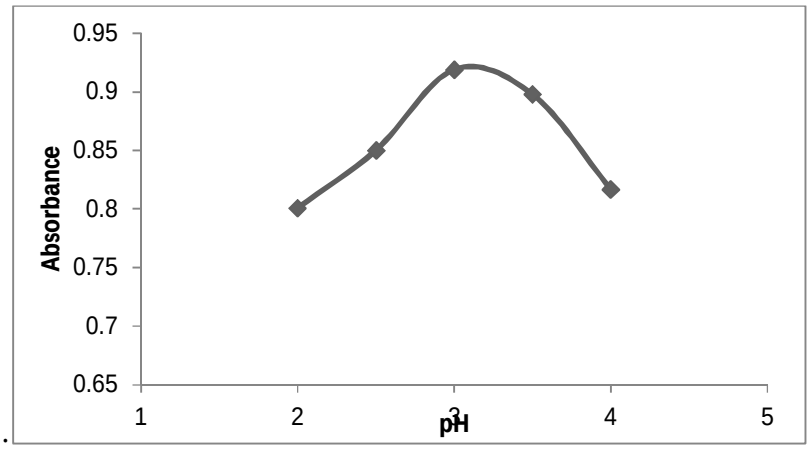


Figure (3.21) Effect of pH on the reaction between esomeprazole and EBT.

3.2.2.2.3.3 Effect of volume of buffer

The effect of volume of buffer was studied in the range of 0.25 to 1.5 ml. Optimum volume was found to be 0.5 ml as in Figure (3.22).

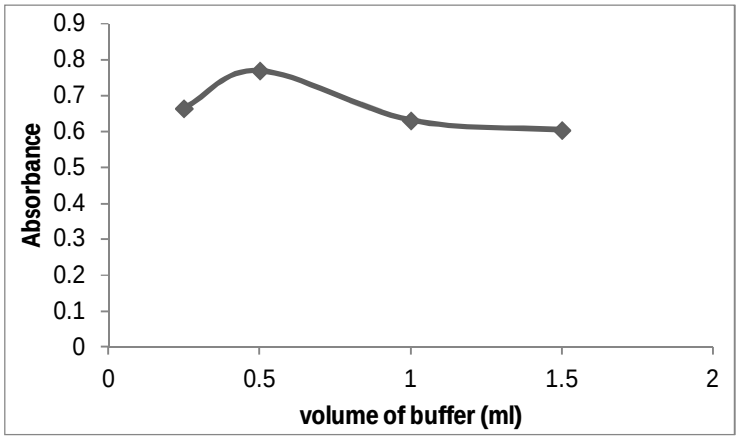


Figure (3.22) Effect of volume of buffer on the reaction between esomeprazole and EBT.

All conditions affecting the reaction were optimized and summarized in Table (3.16).

Table (3.16) Optimum conditions for reaction between esomeprazole and EBT.

Condition	**Optimum**
Reagent concentration % (w/v)	0.5
Volume of reagent (ml)	2.0
pH	3
Volume of buffer (ml)	0.5

Reaction time (min)	Immediately

3.2.2.2.4 Determination of stoichiometry of the reaction

Under optimum conditions, as shown in Table (3.14), the stoichiometry of the reaction between esomeprazole and EBT was investigated by Job's method and was found to be 1:1, as shown in Figure (3.23). The reaction pathway was postulated to proceed as shown in Scheme (3.3).

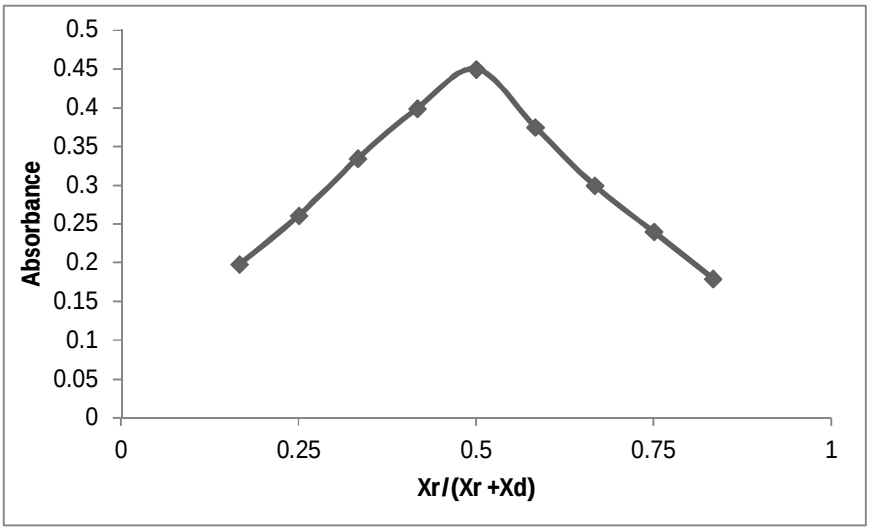
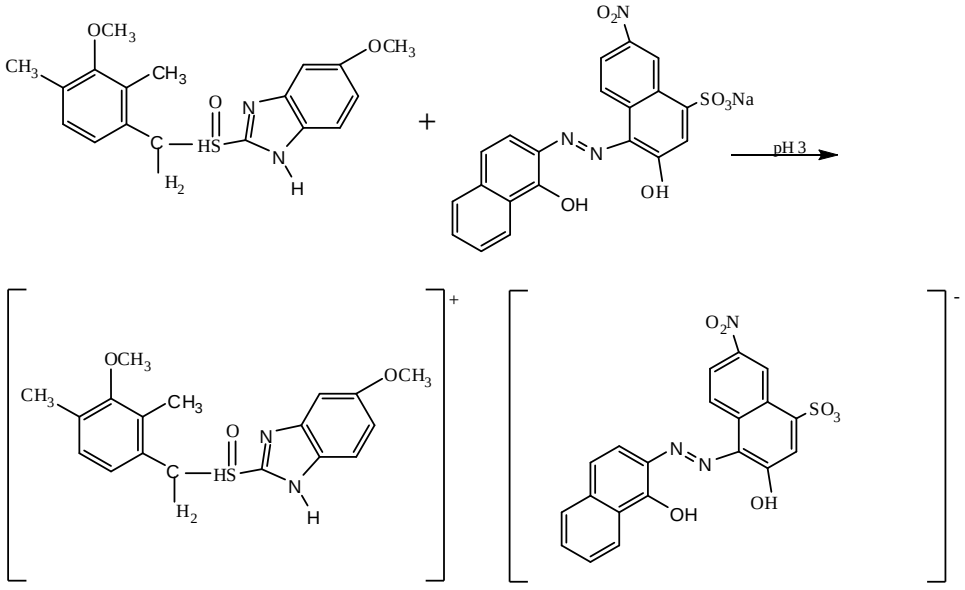


Figure (3.23) Continuous variation plot for stoichiometry of the reaction between esomeprazole and EBT.



Scheme (3.3) Reaction pathway of esomeprazole with EBT.

3.2.2.2.5 Linearity and range

The linearity of the method was investigated and found to be in the range of 1 - 35 µg/ml, as shown in Figure (3.24).

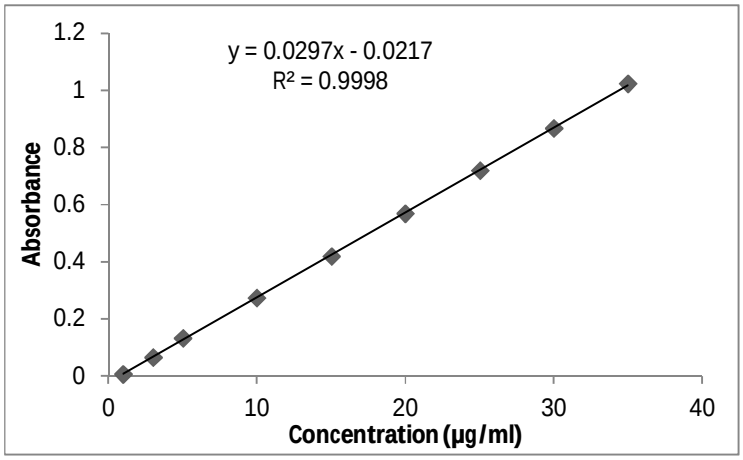


Figure (3.24) Calibration curve for determination of esomeprazole using EBT.

3.2.2.2.6 Precision

To assess the precision, each experiment was repeated three times on the same day (intra-day), and at different days (inter-day). The results show that the method is precise according to the low values of standard deviation (SD), and percent relative standard deviation (% RSD), as shown in Table (3.17).

Table (3.17) Evaluation of intra- and inter-day precision of the proposed spectrophotometric method 2 for assay of esomeprazole magnesium.

Taken (µg/ml)	Intra – day precision			Inter – day precision		
	Found (µg/ml)	SD	% RSD	Found (µg/ml)	SD	% RSD
7	7.08	0.026	0.37	7.13	0.04	0.56
14	13.93	0.025	0.18	13.88	0.056	0.41
21	21.07	0.044	0.21	21.19	0.095	0.45

3.2.2.2.7 Accuracy (Recovery Studies)

Accuracy was estimated in terms of percent recovery and percent relative standard deviation. t-test and F-test values had been calculated using USP standard reference HPLC method. These values were less than their permissible values of: 2.776, and 19.0 for t- and F-test, respectively, at 95% confidence level, indicating a high accuracy and precision of the method as in Table (3.18).

Table (3.18) Accuracy studies of the proposed spectrophotometric method 2 for assay of esomeprazole magnesium.

Taken (µg/ml)	**Found (µg/ml)**	**% Recovery**	**% RSD**	**Proposed Method Mean ± SD**	**Reference Method Mean ± SD**	**t-test**	**F-test**
10	10.05	100.52	0.51	100.15±0.511	99.97 ± 0.416	0.473	1.509
15	15.06	100.37					
20	19.91	99.57					

3.2.2.2.8 Limit of detection and limit of Quantitation

Limit of detection (LOD) and limit of quantification (LOQ) were determined from the regression equation of calibration curves.

LOD = 0.44 µg/ml, LOQ = 1.33 µg/ml.

3.2.2.2.9 Robustness

Robustness was examined by evaluating the influence of small variation in the method variables on its analytical performance. In these experiments, one parameter was changed whereas the others were kept unchanged, and the recovery percentage was calculated each time. It was found that small variation in the method variables did not significantly affect the procedure's recovery values, as shown in Table (3.19).

Table (3.19) Robustness of the proposed spectrophotometric method 2 for assay of esomeprazole magnesium.

Recommended condition	**Recovery (Mean ± SD)***	
Standard	100.15 ± 0.511	
Reagent concentration 0.5%(w/v)	0.45	99.8 ± 0.001
	0.55	99.31 ± 0.0015
λ_{max} (511 nm)	509	100.11 ± 0.002
	513	100.55 ± 0.001
pH (3)	2.8	100.89 ± 0.001
	3.2	99.54 ± 0.01

3.2.2.3 Comparison between the two spectrophotometric methods

All analytical parameters for the two methods for the determination of esomeprazole magnesium were summarized in Table (3.20). It shows that method 2 was more

sensitive than method 1 owing to the lower values of both LOD and LOQ and also higher value of molar absorptivity.

Table (3.20) Analytical Parameters for the determination of esomeprazole using Alizarin Red S and EBT.

Parameter	Value	
	Method 1	**Method 2**
λ_{max} (nm)	521	511
Beer's law limits (µg/ml)	10 – 80	5-35
Sandell's sensitivity (µgcm^{-2})	0.08285	0.03366
Molar absorptivity (dm^{3}mol^{-1}cm^{-1})	9.2597×10^3	2.2788×10^4
Std. Dev. of intercept	0.006484	0.003958
LOD (µg/ml)	1.69	0.44
LOQ (µg/ml)	5.37	1.33
Slope (m)	0.01207	0.029707
Intercept (b)	0.0406	0.02171
Correlation coefficient	0.9999	0.9998
Regression equation	Y = 0.0127 x – 0.0406	Y = 0.0297 x – 0.0217

3.2.2 HPLC method development and validation

3.2.2.1 Development and validation of high-performance liquid chromatography methods for determination of amlodipine besylate: method 1

3.2.2.1.1 Method optimization and regression analysis

To optimize an isocratic RP HPLC method for determination of amlodipine, 2^3 factorial design was used where 3 represents the number of factors affecting the method: percentage of organic modifier, acetonitrile (X_1), percentage of water (X_2), and flow rate (X_3). 2 represents the level of each factor: higher and lower levels. Eight runs were performed and three responses were recorded: w retention time (Y_1), tailing factor (Y_2), and theoretical plates (Y_3). The results obtained are shown in Table (3.21).

Table (3.21) 2^3 factorial design for determination of esomeprazole using HPLC method 1.

Exp NO.	Factors			Responses		
	Acetonitrile % (X_1)	Water % (X_2)	Flow rate (ml/min) (X_3)	Retention time (min) (Y_1)	Tailing factor (Y_2)	Theoretical plates (Y_3)
1	55	35	1.0	5.52	1.44	7508
2	60	35	1.0	5.10	1.54	6321
3	55	40	1.0	6.28	1.27	8760
4	60	40	1.0	6.08	1.48	7367
5	55	35	1.5	3.78	1.36	4447
6	60	35	1.5	3.56	1.58	4556
7	55	40	1.5	4.35	1.46	5087
8	60	40	1.5	3.92	1.36	4808

The above results were statistically analyzed to determine which factor had a significant effect on retention time factor response. Minitab 17 and Microsoft excel were used to analyze the data. Linear regression model was used to illustrate the relationship between a response and a set of process parameters which affect the response. Generally regression model for factors at two levels is usually of the form:

$Y = ß_0+\beta_1 X_1 +\beta_2X_2+\beta_3X_3+ \beta_{1\,2}X_1X_2+ \beta_{2\,3}X_2X_3+ \beta_{1\,3}X_1X_3+ \beta_{1\,2\,3}X_1X_2X_3$

Where: β1, β2 are the regression coefficient, β0 is the average response in a factorial experiment, and β1 2 correspond to interaction between X_1 and X_2.

In this experiment linear regression model with significant term $p \leq 0.05$ (95% confidence level) was used and the adjusted R^2 value obtained from regression analysis model was within the acceptable range (acceptable limit of $R^2 \geq 0.80$), and this indicates that the experimental conditions were a good fit for the equation:

$Y= 4.8237 – 0.1587X_1 + 0.3337X_2 – 0.9213X_3$

With R^2 = 0.9868, adj R^2 = 0.9769, and SD = 0.162211.

Positive sign indicates direct correlation between factor and response, while negative sign indicates inverse correlation.

Two factors were found to be significant (p value < 0.05) for retention time factor Y_1; these factors include X_2 (p = 0.004), and X_3 (p = 0.000). While it is insignificant for X_1 (p = 0.05) as shown in Figure (3.25).

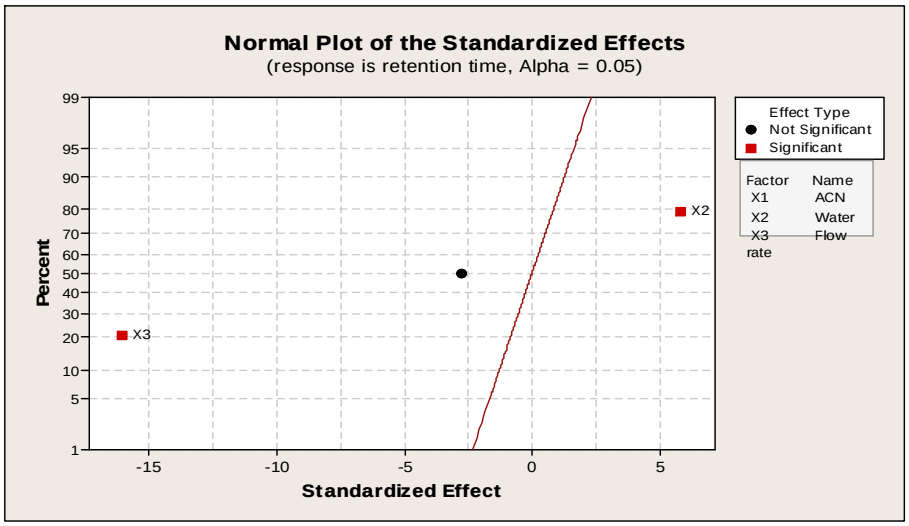


Figure (3.25) Normal plot of standard effect of retention time (Y_1) for HPLC method 1 for assay of amlodipine besylate.

It was found that retention time had decreased by increasing both flow rate and percentage of acetonitrile while it would increases by increasing percentage of water as shown in Figure (3.26).

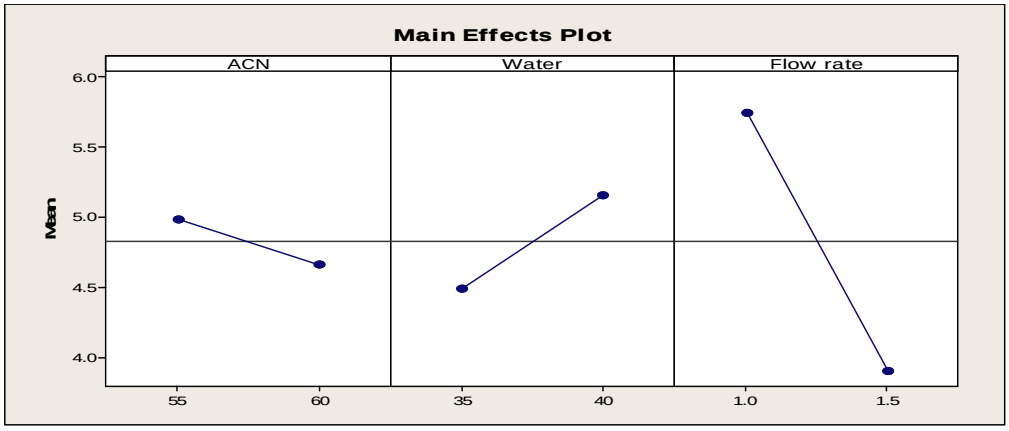


Figure (3.26) The main effect of the three factors affecting retention time at p value 0.05 for HPLC method 1 for assay of amlodipine besylate.

The recommended optimum chromatographic conditions for method 1for determination of amlodipine besylate were summarized in Table (3.22).

Table (3.22) Optimum chromatographic conditions for HPLC method 1 for assay of amlodipine besylate.

Condition	**Optimum**
Column	Gemini Nx C_{18}(110Å, 250×4.6 mm, fully porous silica)
Mobile phase composition	60 :40 ACN:water with 0.1% TEA
Flow rate	1.0 ml/min
Detection wavelength	362 nm
Injection volume	35µL
Temperature	Ambient

3.2.2.1.2 System suitability

The result for system suitability was obtained by injecting one concentration six times, and then percentage RSD was calculated as described in Table (3.23).

Table (3.23) System suitability data for determination of amlodipine besylate using HPLC method 1.

Exp. No.	Retention time (min)	Peak area	Tailing factor	Theoretical plates
1	5.775	612445	1.212	7141
2	5.773	614896	1.211	7270
3	5.774	617227	1.213	7235
4	5.774	615484	1.215	7183
5	5.774	617435	1.213	7187
6	5.771	617552	1.215	7204
Mean	5.7735	615839.8	1.213167	7203.333
%RSD	0.023875	0.324294	0.132058	0.621256
Limit	NMT 1%	NMT 2%	NMT 2%	NLT 2000

3.2.2.1.3 Linearity and range

Linearity of this method was found to be in range from 2.5 to 50 µg/ml as described in Figure (3.27).

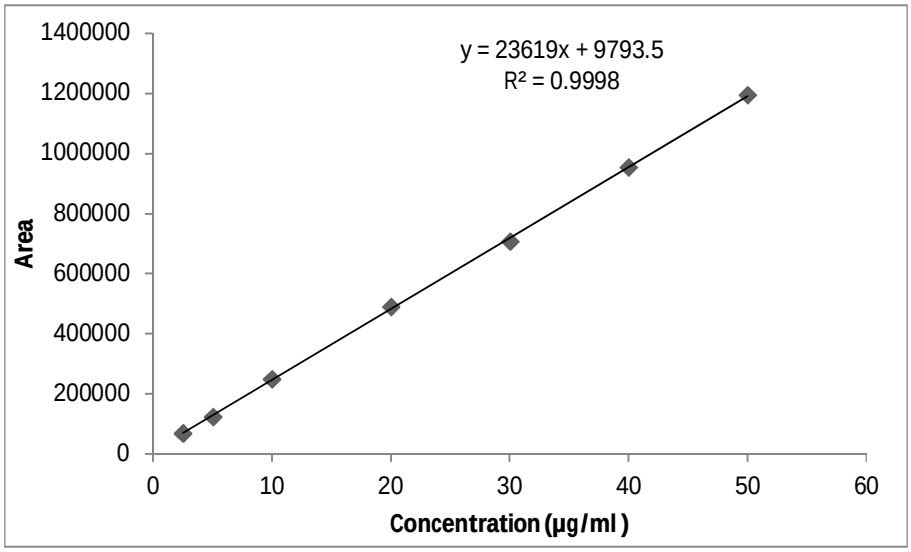


Figure (3.27) Calibration curve for determination of amlodipine using HPLC method 1.

3.2.2.1.4 Precision

Intra-day and inter-day precision for determination of amlodipine besylate using HPLC method 1 were studied and results show that the method is precise according to the low values of standard deviation (SD), and percent relative standard deviation (% RSD), as shown in Table (3.24).

Table (3.24) Evaluation of intra- and inter-day precision of the proposed HPLC method 1 for assay of amlodipine besylate.

Taken (µg/ml)	**Intra – day precision**			**Inter – day precision**			**Accepted criteria**
	Found (µg/ml)	**SD**	**% RSD**	**Found (µg/ml)**	**SD**	**% RSD**	
15	14.90	0.0133	0.0896	14.90	0.010	0.067	%RSD ≤ 2%
25	25.17	0.0682	0.271	25.15	0.127	0.504	
35	37.08	0.0510	0.145	35.03	0.050	0.143	

3.2.2.1.5 Recovery (accuracy)

Accuracy was estimated in terms of percent recovery and percent relative standard deviation. t-test and F-test values had been calculated using USP standard reference

HPLC method. The t-test and F-test values were less than their permissible values of: 2.776, and 19.0 for t- and F-test, respectively, at 95% confidence level, indicating a high accuracy and precision of the method as shown in Table (3.25).

Table (3.25) Accuracy studies of the proposed HPLC method 1 for assay of amlodipine besylate.

Taken (µg/ml)	**Found (µg/ml)**	**% Recovery**	**% RSD**	**Proposed Method Mean ± SD**	**Reference Method Mean ± SD**	**Accepted criteria**	**t-test**	**F-test**
15	15.08	100.542	0.293	100.27 ± 0.294	99.77 ±0.30	98 -102	1.052	6.75
30	30.09	100.312						
45	44.98	99.96						

3.2.2.1.6 Sensitivity

Sensitivity of the method was investigated by measurement of limit of detection (LOD), and limit of quantification (LOQ), which were found to be 0.592 and 1.793 µg/ml respectively. Their values proved that the proposed method was sensitive for quantification of amlodipine besylate.

3.2.2.1.7 Selectivity

Method was found to be selective for amlodipine since it gave no response for placebo solution as shown in Figure (3.28).

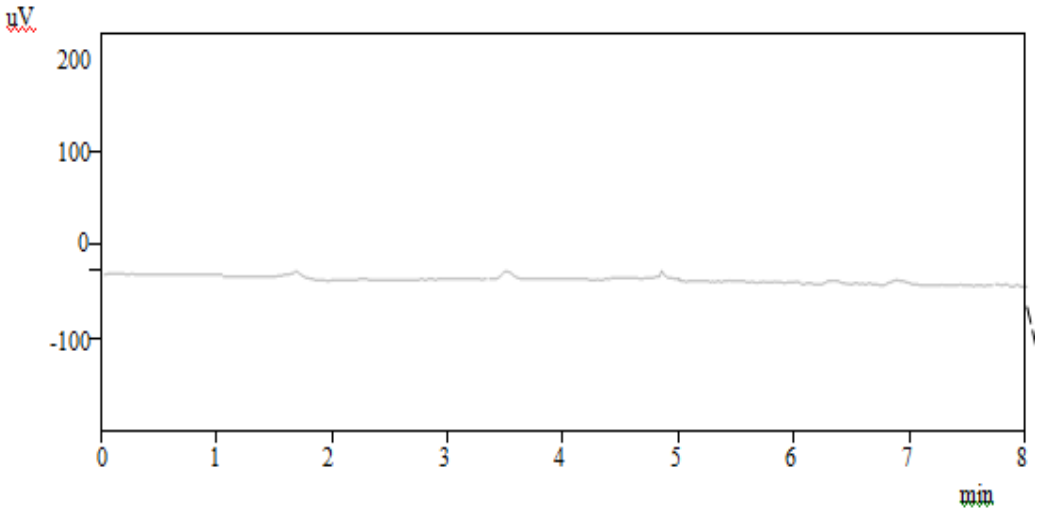


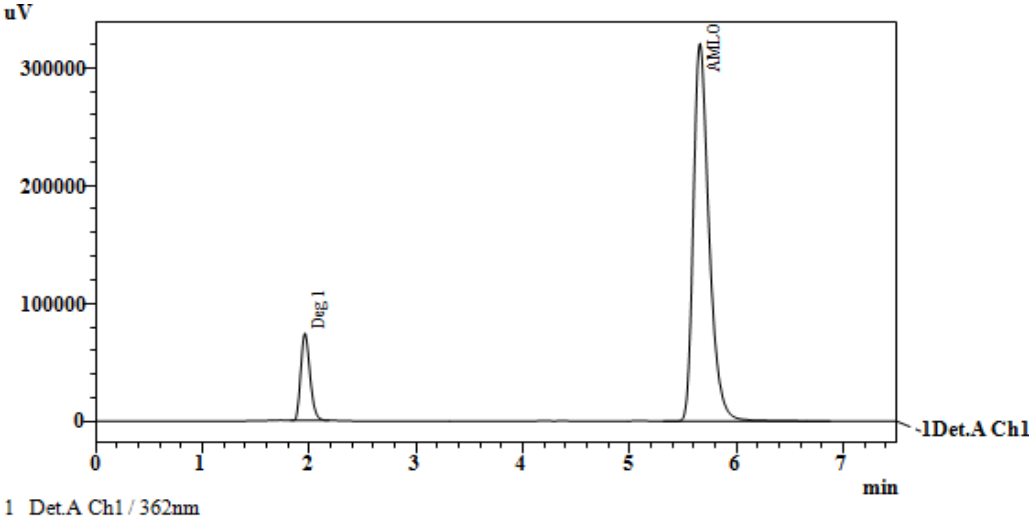
Figure (3.28) Chromatogram of placebo solution for amlodipine besylate using HPLC method 1.

3.2.2.1.8 Specifity

To study specifity of the proposed method, stress degradation was performed using different stress conditions, such as, acid, base, oxidation, thermal and photolytic as shown in Table (3.26). Under basic stress condition, one degradation product was detected as shown in Figure (3.29), and under oxidation stress condition, one degradation product was detected as shown in Figure (3.30).

Table (3.26) Degradation study of amlodipine under various conditions using HPLC method 1.

No.	Condition	Assay %
1	Acid	92.66
2	Base	79.89
3	Oxidation	86.75
4	Thermal	94.84

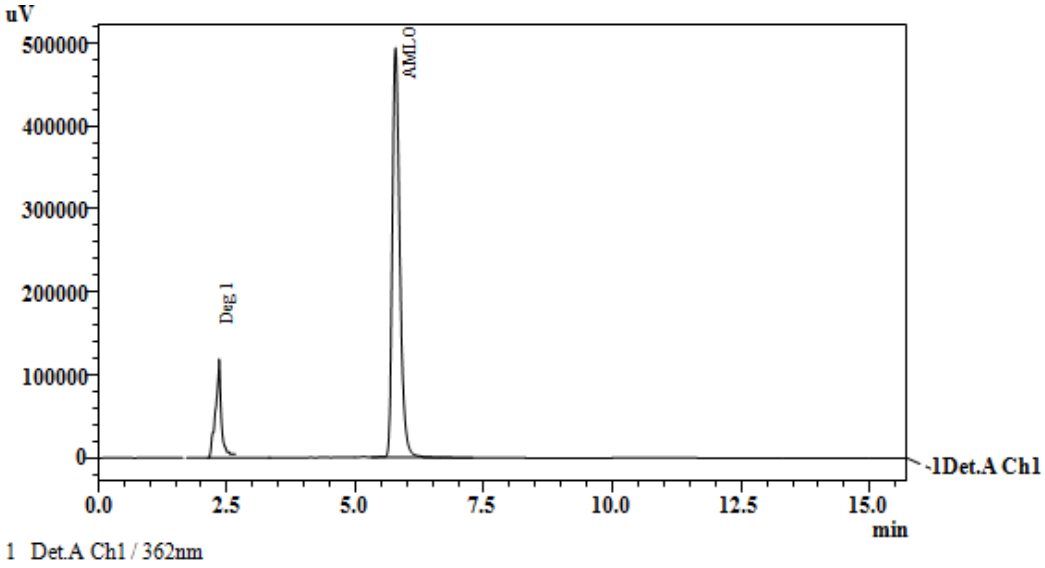


PeakTable

Detector A Ch1 362nm

Name	Ret. Time	Area	Resolution	Tailing Factor	Theoretical Plate#
Deg 1	1.954	438427	0.000	1.311	2100.045
AMLO	5.651	3269174	16.793	1.429	7019.477
		3707601			

Figure (3.29) Chromatogram of base stress degradation of amlodipine besylate using HPLC method 1.



PeakTable

Detector A Ch1 362nm

Name	Ret. Time	Area	Resolution	Tailing Factor	Theoretical Plate#
Deg 1	2.347	205389	0.000	0.936	2226.715
AMLO	5.777	4976579	7.707	1.390	7501.211
		5181968			

Figure (3.30) Chromatogram of oxidation stress degradation of amlodipine besylate using HPLC method 1.

3.2.2.1.9 Robustness

Robustness of the method was measured by the capability of the method to remain unaffected by small variation in method parameters. For this measurement effect of changing three factors were studied as shown in Tables (3.27, 3.28, and 3.29).

Table (3.27) Effect of changing mobile phase composition on the performance of HPLC method 1 for the assay of amlodipine besylate.

Factor			Response				Accepted criteria
Mobile phase composition			R_t (min)	% RSD	Area	% RSD	%RSD ≤ 2%
Original	Level	Used					
60 : 40	-1	59 : 41	5.877	0.329	417663	0.370	
	0	60 : 40	5.774	0.017	604275	0.267	
	+1	61 : 39	5.856	0.491	591308	5.2×10^{-2}	

Table (3.28) Effect of changing wavelength of detection on the performance of HPLC method 1 for the assay of amlodipine besylate.

Factor			Response				Accepted criteria
Wavelength of detection (nm)			R_t (min)	% RSD	Area	% RSD	%RSD ≤ 2%
Original	Level	Used					
362	-1	360	5.791	0.036	605729	0.333	
	0	362	5.774	0.017	604275	0.267	
	+1	364	5.795	1.88×10^{-14}	607593	0.434	

Table (3.29) Effect of changing flow rate on the performance of HPLC method 1 for the assay of amlodipine besylate.

Factor			Response				Accepted criteria
Flow rate (ml/min)			R_t (min)	% RSD	Area	% RSD	%RSD ≤ 2%
Original	Level	Used					
1.0	-1	0.9	6.482	0.775	675263	0.071	
	0	1	5.774	0.017	604275	0.267	
	+1	1.1	5.278	0.054	552899	0.722	

3.2.2.2 Development and validation of high performance liquid chromatography methods for determination of amlodipine besylate: method 2

3.2.2.2.1 Method optimization

Optimum conditions for this method were summarized in Table (3.30).

Table (3.30) Optimum chromatographic conditions for HPLC method 2 for assay of amlodipine besylate.

Condition	**Optimum**
Column	Synergi hydro end capped C_{18} (80Å, 150×4.6 mm)
Mobile phase composition	20 mM KH_2PO_4
Flow rate	1.5 ml/min
Detection wavelength	212 nm
Injection volume	35µL
Temperature	Ambient

3.2.2.2.2 System suitability

The result for system suitability was obtained by injecting one concentration six times; then %RSD was calculated as shown in Table (3.31).

Table (3.31) System suitability data for determination of amlodipine besylate using HPLC method 2.

Exp. No.	Retention time (min)	Peak area	Tailing factor	Theoretical plates
1	4.926	999521	1.482	3584
2	4.946	993624	1.480	3550
3	4.960	997264	1.490	3554
4	4.978	996064	1.476	3597
5	4.971	999846	1.485	3575
6	4.942	997967	1.473	3542
Mean	4.954	997381	1.481	3567
%RSD	0.393	0.232	0.408	0.606
Limit	NMT 1%	NMT 2%	NMT 2%	NLT 2000

3.2.2.2.3 Linearity and range

Linearity of this method was found to lie in range of 5 – 60 µg/ml as shown in Figure (3.31).

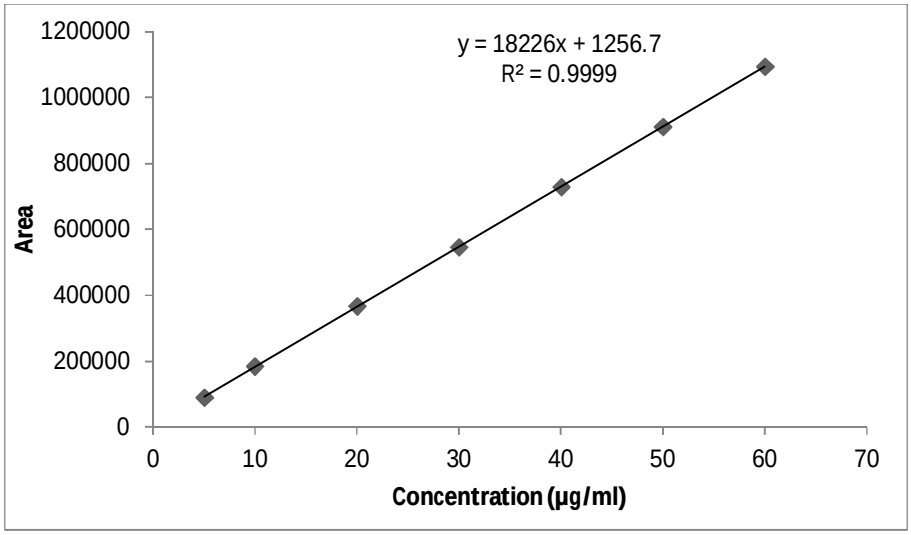


Figure (3.31) Calibration curve for determination of amlodipine using HPLC method 2.

3.2.2.2.4 Precision

Intra-day and inter-day precision for determination of amlodipine besylate using HPLC method 2were studied and results show that the method is precise according to the low values of standard deviation (SD), and percent relative standard deviation (% RSD), as shown in Table (3.32).

Table (3.32): Evaluation of intra- and inter-day precision of the proposed HPLC method 1 for assay of amlodipine besylate.

Taken (µg/ml)	**Intra – day precision**			**Inter – day precision**			**Accepted criteria**
	Found (µg/ml)	**SD**	**% RSD**	**Found (µg/ml)**	**SD**	**% RSD**	
20	20.061	0.117	0.584	20.042	0.082	0.410	%RSD ≤ 2%
30	30.058	0.126	0.419	30.153	0.083	0.275	
40	39.978	0.031	0.076	39.968	0.105	0.026	

3.2.2.2.5 Recovery (accuracy)

Accuracy was estimated in terms of percent recovery and percent relative standard deviation. t-test and F-test values had been calculated using USP standard reference HPLC method. Their calculated values were less than their permissible values of: 2.776 and 19.0 for t- and F-test, respectively, at 95% confidence level, indicating a high accuracy and precision of the method as shown in Table (3.33).

Table (3.33) Accuracy studies of the proposed HPLC method 2 for assay of amlodipine besylate.

Taken (µg/ml)	**Found (µg/ml)**	**% Recovery**	**% RSD**	**Proposed Method Mean ± SD**	**Reference Method Mean ± SD**	**Accepted criteria**	**t-test**	**F-test**
45	45.51	101.13	0. 685	100.43 ± 0.685	99.77± 0.30	98 -102	1.10	1.23
55	55.23	100.39						
57	64.85	99.76						

3.2.2.2.6 Sensitivity

Sensitivity of the method was investigated by measurement of limit of detection (LOD), and limit of quantification (LOQ). LOD and LOQ were found to be 0.254 and 0.770

µg/ml respectively. Their values proved that the proposed method was sensitive for quantification of amlodipine besylate.

3.2.2.2.7 Selectivity

Method 2 was found to be selective for amlodipine since it gave no response for placebo solution as shown in Figure (3.32).

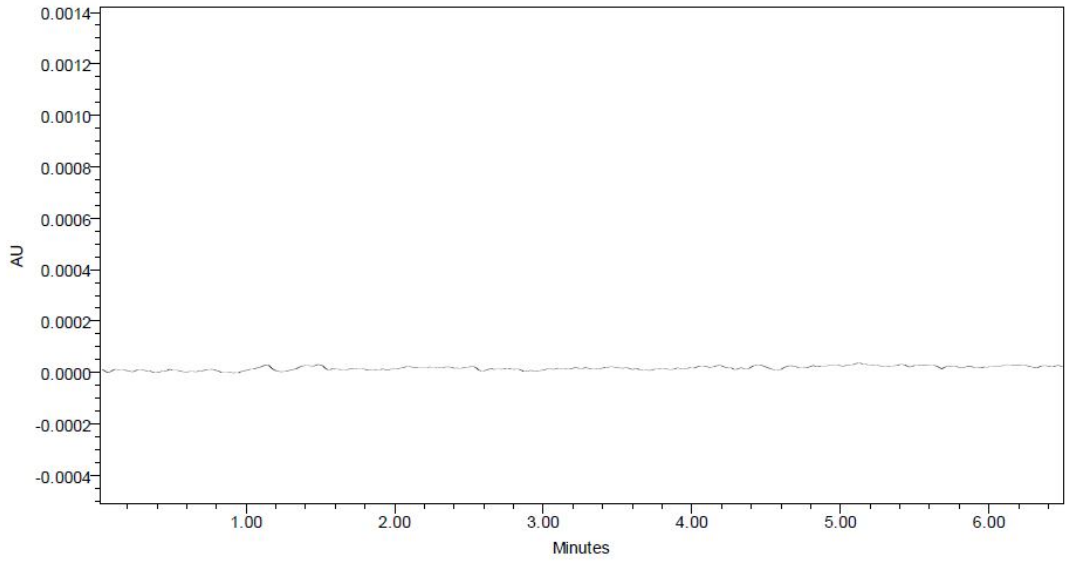


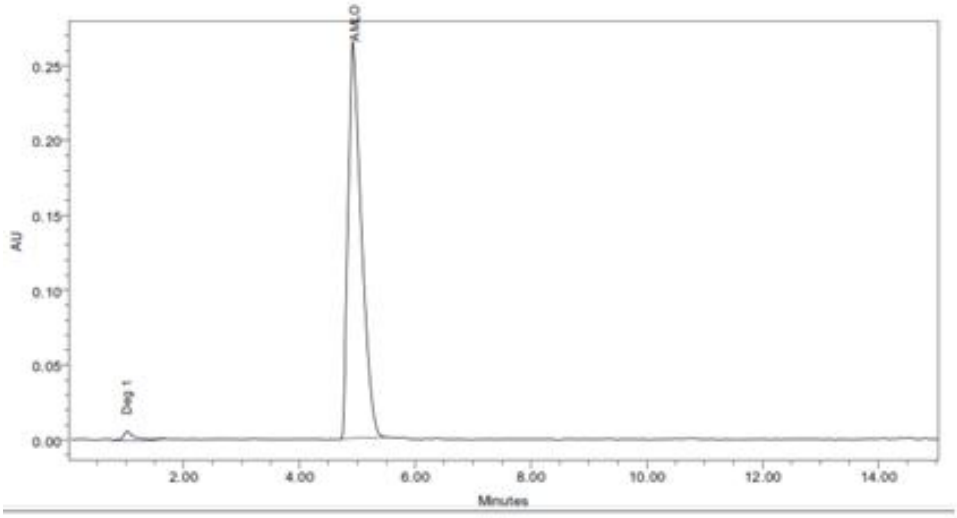
Figure (3.32) Chromatogram of placebo solution for amlodipine besylate using HPLC method 2.

3.2.2.2.8 Specifity

To study specifity of the proposed method, stress degradation was performed using different stress conditions, such as, acid, base, oxidation, thermal and photolytic as shown in Table (3.34). Under basic stress condition, one degradation product was detected as in Figure (3.33), and, under oxidation stress condition, one degradation product was detected as in Figure (3.34).

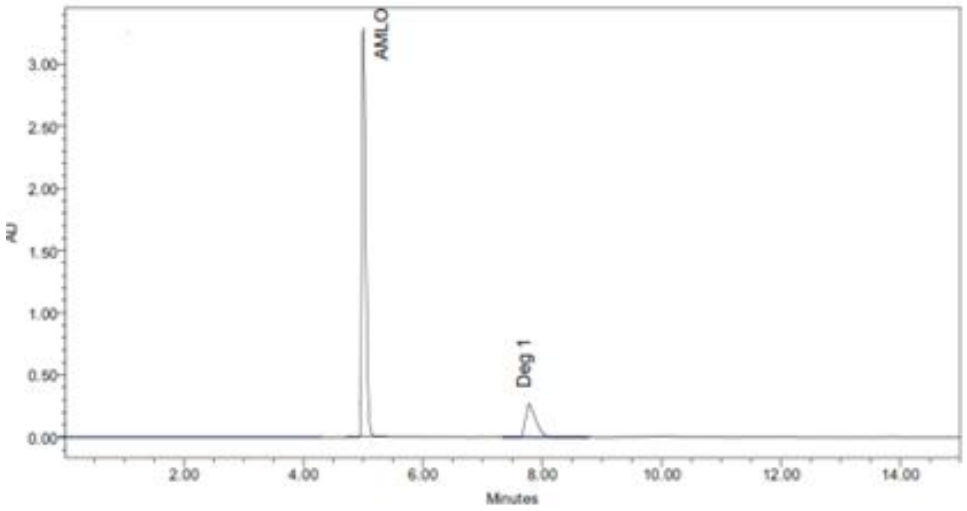
Table (3.34) Degradation study of amlodipine under various conditions.

No.	Condition	Assay %
1	Acid	91.99
2	Base	77.72
3	Oxidation	85.10
4	Thermal	93.38



Name	RT	Area	Resolution	Tailing factor	Plate count
Deg 1	1.159	14010.7	0.00	1.452	2010.2
AMLO	4.924	420319.9	7.52	1.338	3584.4

Figure (3.33) Chromatogram of base stress degradation of amlodipine besylate using HPLC method 2.



Name	RT	Area	Resolution	Tailing factor	Plate count
AMLO	4.931	423885.1	0.00	1.452	3609.8
Deg 1	7.898	20955.9	5.97	1.338	4574.2

Figure (3.34) Chromatogram of oxidation stress degradation of amlodipine besylate using HPLC method 1.

3.2.2.2.9 Robustness

Robustness of the method was measured by the capability of the method to remain unaffected by small variation in method parameters. To measure robustness two factors were studied as shown in Tables (3.35) and (3.36).

Table (3.35) Effect of changing wavelength of detection on the performance of HPLC method 2 for the assay of amlodipine besylate.

Factor			Response				Accepted criteria
Wavelength of detection (nm)			R_t (min)	% RSD	Area	% RSD	%RSD ≤ 2%
Original	Level	Used					
212	-1	210	5.000	0.031	967443	0.156	
	0	212	5.003	0.060	963286	0.093	
	+1	214	4.988	0.200	965033	0.105	

Table (3.36) Effect of changing flow rate on the performance of HPLC method 2 for the assay of amlodipine besylate.

Factor			Response				Accepted criteria
Flow rate (ml/min)			R_t (min)	% RSD	Area	% RSD	
Original	Level	Used					
1.5	-1	1.35	5.368	0.398	1015596	0.456	%RSD ≤ 2%
	0	1.5	5.003	0.060	963286	0.093	
	+1	1.65	4.781	0.085	946795	0.201	

3.2.2.3 Development and validation of high performance liquid chromatography methods for determination of esomeprazole magnesium: method 1

3.2.2.3.1 Method optimization and regression analysis

To optimize an isocratic RP-HPLC method for determination of esomeprazole, 2^3 factorial design was used, 3 represents the number of factors affecting the method: percentage of organic modifier, methanol (X_1), percentage of water (X_2), and flow rate (X_3). 2 represents the level of each factor: higher and lower levels. Eight runs were performed and three responses were recorded which were retention time (Y_1), tailing factor (Y_2), and theoretical plates (Y_3). The results obtained were shown in Table (3.37).

Table (3.37) Shows 2^3 factorial design for determination of esomeprazole using HPLC method 1.

Exp NO.	Factors			Responses		
	Acetonitrile % (X_1)	Water % (X_2)	Flow rate (ml/min) (X_3)	Retention time (min) (Y_1)	Tailing factor (Y_2)	Theoretical plates (Y_3)
1	60	40	1.0	5.409	1.214	4728
2	65	40	1.0	5.251	1.220	4633
3	60	45	1.0	7.686	1.132	5942
4	65	45	1.0	6.494	1.168	5379
5	60	40	1.2	4.544	1.189	4282
6	65	40	1.2	4.428	1.197	4226
7	60	45	1.2	6.435	1.115	5390
8	65	45	1.2	5.418	1.145	4864

Linear regression model with significant term $p \leq 0.05$ (95% confidence level) was used, in this experiment, and the adjusted R^2 value obtained from regression analysis model was within the acceptable range (acceptable limit of $R^2 \geq 0.80$), and these values indicated that the experimental conditions were a good fit for the equation:

$Y = 1.1725 + 0.01\ X_1 - 0.0325X_2 - 0.011X_3$

With $R^2 = 0.9663$, adj $R^2 = 0.9411$, and SD = 0.0694.

All factors were found to be significant (p value < 0.05) for tailing factor Y_2 as shown in Figure (3.35).

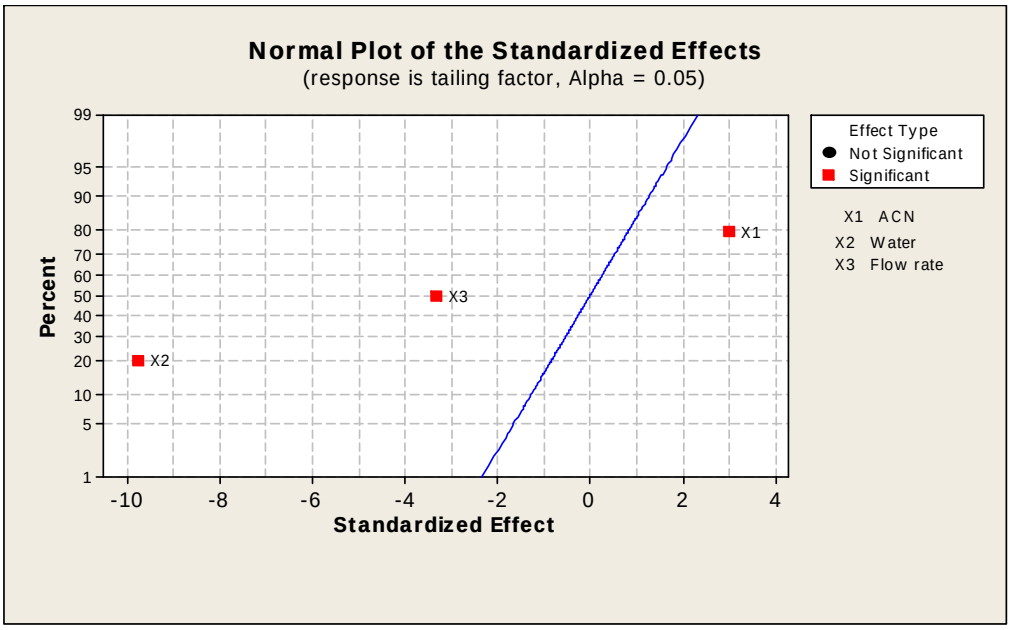


Figure (3.35) Normal plot of the standard effect for tailing factor (Y_2) for HPLC method 1 for assay of esomeprazole.

Tailing factor response would be decreased by increasing both X_2 and X_3, would increasing X_1 would lead to increase in tailing factor response as shown in Figure (3.36).

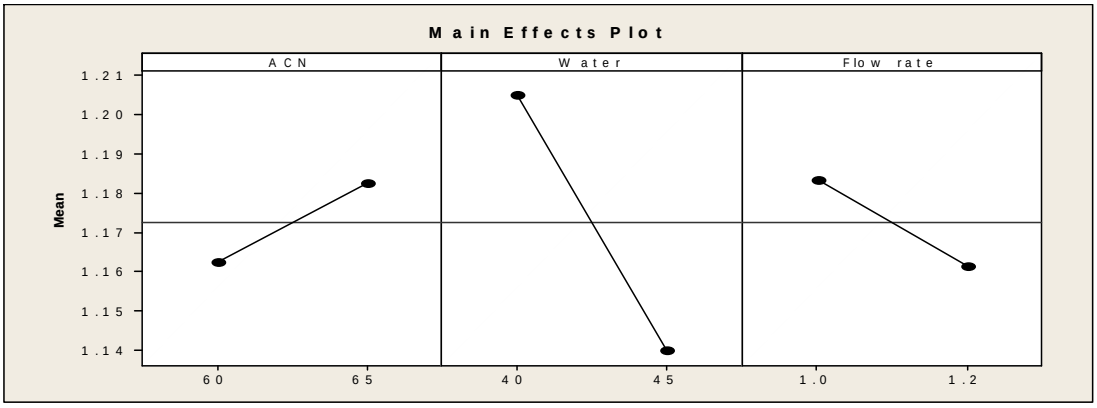


Figure (3.36) The main effect of the three factors affecting tailing factor at p value 0.05 for HPLC method 1 for assay of esomeprazole.

The recommended optimized chromatographic conditions for HPLC method 1 for assay of esomeprazole were summarized in Table (3.38).

Table (3.38) Optimum chromatographic conditions for HPLC method 1 for assay of esomeprazole.

Condition	**Optimum**
Column	Densyl bonded C_{12} (150×4.5 mm, 5µm, 80 Å)
Mobile phase composition	60:40 (MeOH:water)
Flow rate	1.0 ml/min
Detection wavelength	302 nm
Injection volume	10 µL
Temperature	Ambient

3.2.2.3.2 System suitability

The result for system suitability was obtained by injecting one concentration six times, and then percentage RSD was calculated as described in Table (3.39).

Table (3.39) System suitability data for determination of esomeprazole using HPLC method 1.

Exp. No.	Retention time (min)	Peak area	Tailing factor	Theoretical plates
1	5.308	234753	1.27	4937
2	5.318	236037	1.27	4960
3	5.323	237086	1.268	4960
4	5.331	239425	1.269	4971
5	5.333	237440	1.271	4943
6	5.342	238227	1.267	4997
Mean	5.326	237161	1.269	4961
%RSD	0.226	0.690	0.116	0.432
Limit	NMT 1%	NMT 2%	NMT 2%	NLT 2000

3.2.2.3.3 Linearity and range

Linearity of this method was found to be in range from 5 to 50 µg/ml as shown in figure (3.37).

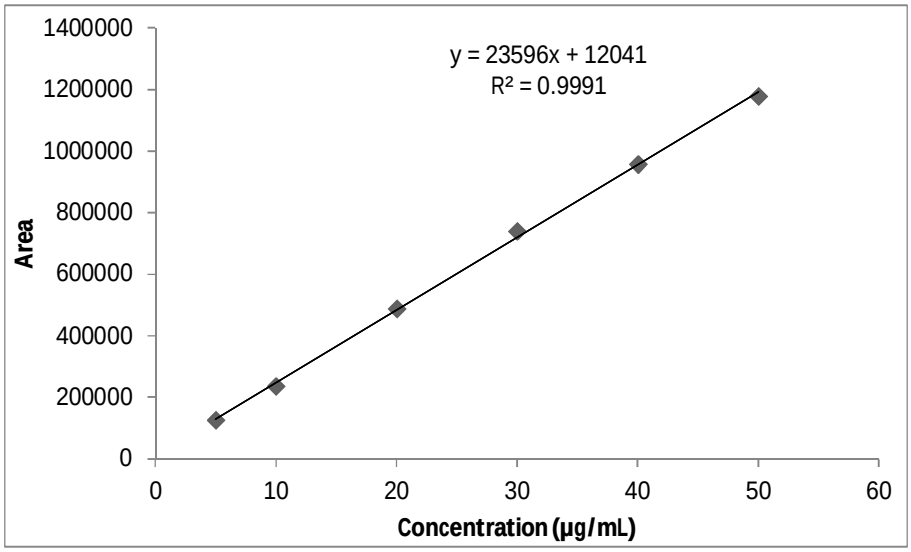


Figure (3.37) Calibration curve for determination of esomeprazole using HPLC method 1.

3.2.2.3.4 Precision

Intra-day and inter-day precision for determination of esomeprazole using HPLC method 1were studied and results show that the method is precise according to the low values of standard deviation (SD), and percent relative standard deviation (% RSD), as shown in Table (3.40).

Table (3.40) Evaluation of intra- and inter-day precision for the propose HPLC method 1 for assay of esomeprazole.

Taken (µg/ml)	**Intra – day precision**			**Inter – day precision**			**Accepted criteria**
	Found (µg/ml)	**SD**	**% RSD**	**Found (µg/ml)**	**SD**	**% RSD**	
9.5	9.5399	0.021	0.22	9.5577	0.016	0.17	%RSD ≤ 2%
19.5	19.4716	0.006	0.030	19.4629	0.008	0.039	
29.5	29.6893	0.025	0.083	29.6937	0.031	0.103	

3.2.2.3.5 Recovery (accuracy)

Accuracy was estimated in terms of percent recovery and percent relative standard deviation. Values of t-test and F-test had been calculated using USP standard reference

HPLC method. These values were less than their permissible values of: 2.776 and 19.0 for t- and F-test, respectively, at 95% confidence level, indicating a high accuracy and precision of the method as shown in Table (3.41).

Table (3.41) Accuracy studies of the proposed HPLC method 1 for assay of esomeprazole.

Taken (µg/ml)	**Found (µg/ml)**	**% Recovery**	**% RSD**	**Proposed Method Mean ± SD**	**Reference Method Mean ± SD**	**Accepted criteria**	**t-test**	**F-test**
25	24.975	99.90	0.180	100.09 ± 0.180	99.97± 0.416	98 -102	0.841	1.156
35	35.038	100.11						
45	45.117	100.26						

3.2.2.3.6 Sensitivity

Sensitivity of the method was investigated by measurement of limit of detection (LOD), and limit of quantification (LOQ), which was found to be 1.52 and 4.61 µg/ml respectively. These values proved that the proposed method is sensitive for quantification of esomeprazole.

3.2.2.3.7 Selectivity

Method was found to be selective for esomeprazole since it gave no response for placebo solution as shown in Figure (3.38).

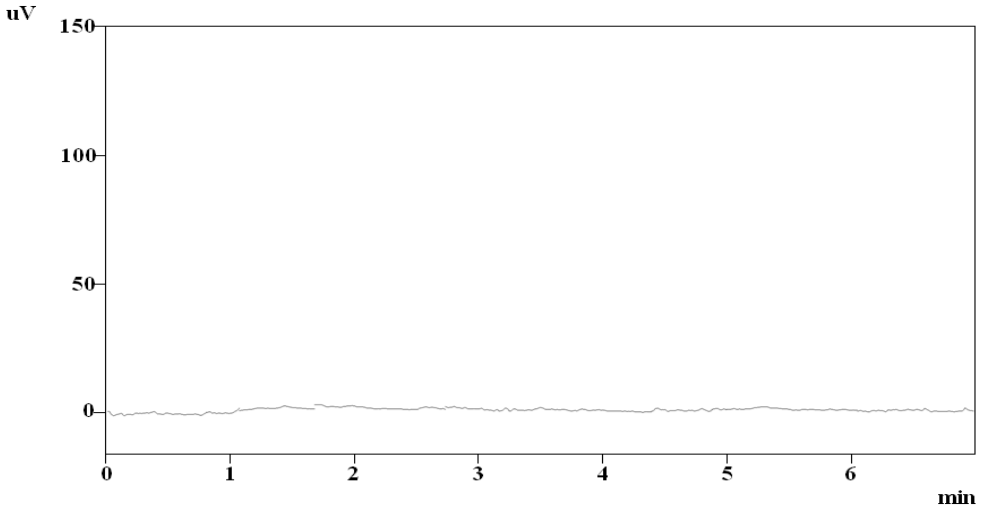


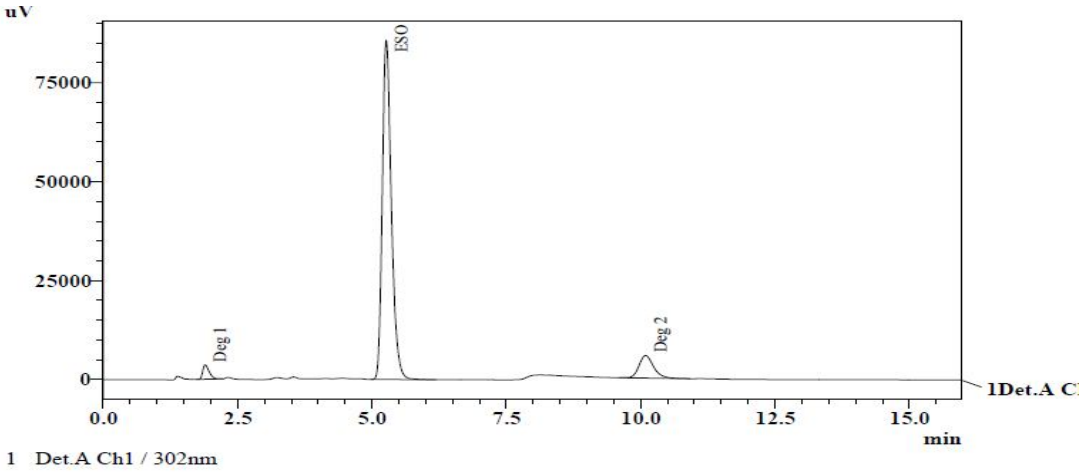
Figure (3.38) Chromatogram of placebo solution for esomeprazole using HPLC method 1.

3.2.2.3.8 Specifity

To study Specifity of the propose method, stress degradation was performed using different stress conditions, such as, acid, base, oxidation, thermal and photolytic as shown in Table (3.42). Under acidic stress condition, two degradation products were detected as shown in Figure (3.39), under oxidation stress condition, two degradation products were detected, as shown in Figure (3.40), while under photolytic stress condition, three degradation products were detected as shown in Figure (3.41).

Table (3.42) Degradation study of esomeprazole under various conditions using HPLC method 1.

No.	Condition	Assay %
1	Acid	50.85
2	Base	93.42
3	Oxidation	71.53
4	Thermal	96.48

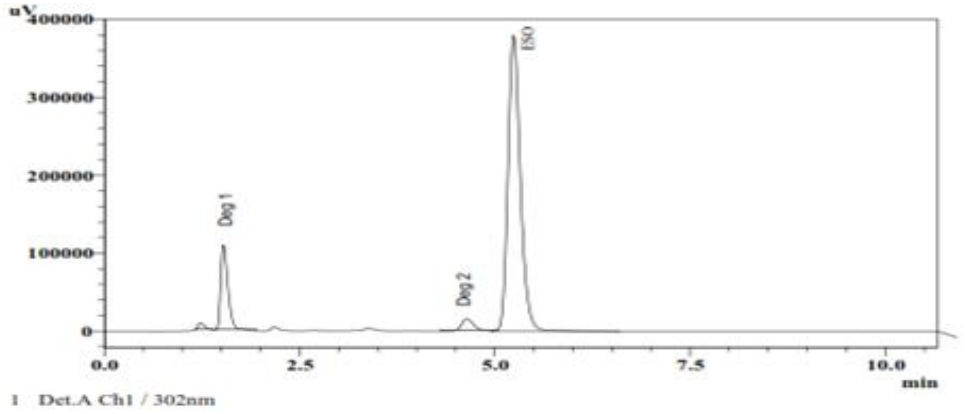


PeakTable

Detector A Ch1 302nm

Name	Ret. Time	Area	Resolution	Tailing Factor	Theoretical Plate#
Deg 1	1.894	29545	0.000	1.614	1125.468
ESO	5.264	1006636	12.555	1.338	4589.436
Deg 2	10.089	106885	12.124	1.267	6917.723
		1143066			

Figure (3.39) Chromatogram of acid stress degradation of esomeprazole using HPLC method 1.

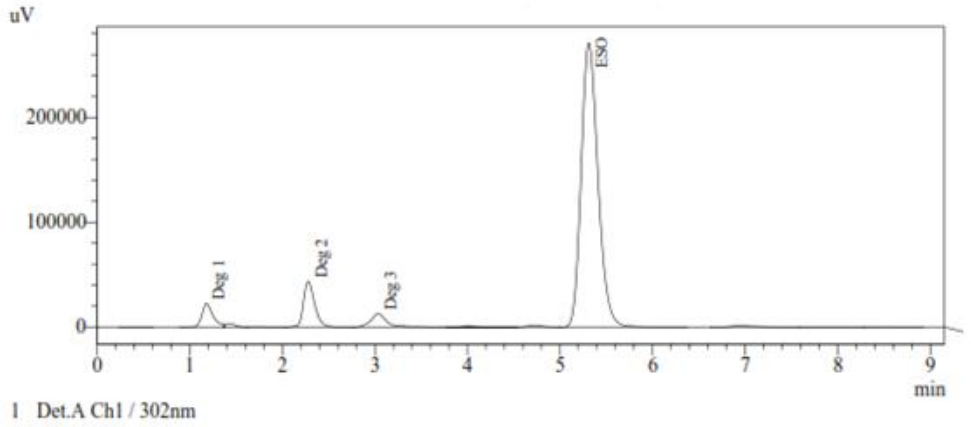


PeakTable

Detector A Ch1 302nm

Name	Ret. Time	Area	resolution	Tailing Factor	Theoretical Plate#
Deg 1	1.559	737605	0.0000	1.476	2321.93
Deg 2	4.826	289654	12.00	1.361	4505.96
AMLO	6.435	3616202	2.074	1.248	5248.08
		4640461			

Figure (3.40) Chromatogram of oxidation stress degradation of esomeprazole using HPLC method 1.



PeakTable

Detector A Ch1 302nm

Name	Ret. Time	Area	Resolution	Tailing Factor	Theoretical Plate #
Deg 1	1.178	191152	0.00	0.975	2445.80
Deg 2	2.278	373868	3.80	1.394	2628.47
Deg 3	3.031	163399	2.95	1.358	2810.15
ESO	5.307	3420460	6.86	1.240	4007.95
		4148879			

Figure (3.41) Chromatogram of photolytic stress degradation of esomeprazole using HPLC method 1.

3.2.2.3.9 Robustness

Robustness of the method was measured by the capability of the method to remain unaffected by small variation in method parameters. For this measurement effects of changing three factors were studied: mobile phase composition, wavelength of detection and flow rate, as shown in Tables (3.43), (3.44), and (3.45).

Table (3.43) Effect of changing mobile phase composition on the performance of HPLC method 1 for the assay of esomeprazole.

Factor			Response				Accepted criteria
Mobile phase composition			R_t (min)	% RSD	Area	% RSD	
Original	Level	Used					
60 : 40	-1	59 : 41	5.666	0.020	217399	0.060	%RSD ≤ 2%
	0	60 : 40	5.306	0.050	217601	0.148	
	+1	61 : 39	5.022	0.166	217363	0.044	

Table (3.44) Effect of changing wavelength of detection for the assay of esomeprazole.

Factor			Response				Accepted criteria
Wavelength of detection (nm)			R_t (min)	% RSD	Area	% RSD	
Original	Level	Used					
302	-1	300	5.303	0.047	217472	0.150	%RSD ≤ 2%
	0	302	5.306	0.050	217601	0.148	
	+1	304	5.309	0.107	217551	0.264	

Table (3.45) Effect of changing flow rate for the assay of esomeprazole.

Factor			Response				Accepted criteria
Flow rate (ml/min)			R_t (min)	% RSD	Area	% RSD	
Original	Level	Used					
1.0	-1	0.9	5.890	0.039	236641	0.175	%RSD ≤ 2%
	0	1	5.306	0.050	217601	0.148	
	+1	1.1	4.837	0.075	201572	0.102	

3.2.2.4 Development and Validation of High performance Liquid Chromatography Methods for determination of esomeprazole magnesium: method 2

3.2.2.4.1 Method optimization and regression analysis

To optimize an isocratic RP-HPLC method for determination of esomeprazole, 2^3 factorial design was used. 3 represents the number of factors affecting the method which were: percentage of organic modifier, ethanol(X_1), percentage of water (X_2), and flow rate (X_3). 2 represents the level of each factor: higher and lower levels. Eight runs were performed and three responses were recorded which were retention time (Y_1), tailing factor (Y_2), and height (Y_3). The results obtained were shown in Table (3.46).

Table (3.46) Shows 2^3 factorial design for determination of esomeprazole using HPLC method 2.

Exp NO.	Factors			Responses		
	Ethanol % (X_1)	Water % (X_2)	Flow rate (ml/min) (X_3)	Retention time (min) (Y_1)	Tailing factor (Y_2)	Height (Y_3)
1	55	45	1.0	7.255	1.376	75.197
2	60	45	1.0	7.164	1.254	71.166
3	55	50	1.0	8.453	1.320	59.159
4	60	50	1.0	7.799	1.200	65.513
5	55	45	1.5	6.288	1.256	68.597
6	60	45	1.5	6.114	1.454	65.207
7	55	50	1.5	7.220	1.350	54.390
8	60	50	1.5	6.582	1.230	59.890

In this experiment, linear regression model with significant term $p \leq 0.05$ (95% confidence level) was used and the adjusted R^2 value obtained from regression analysis model was within the acceptable range (acceptable limit of $R^2 \geq 0.80$), indicating that the experimental conditions were a good fit for the equation:

$Y = 6.70 - 0.0778\ X_1 + 0.1616X_2 – 2.234X_3$

With $R^2 = 0.9631$, adj $R^2 = 0.9355$, and SD = 0.1982.

All factors were found to be significant (p value < 0.05) for retention time Y_1, as shown in Figure (3.42).

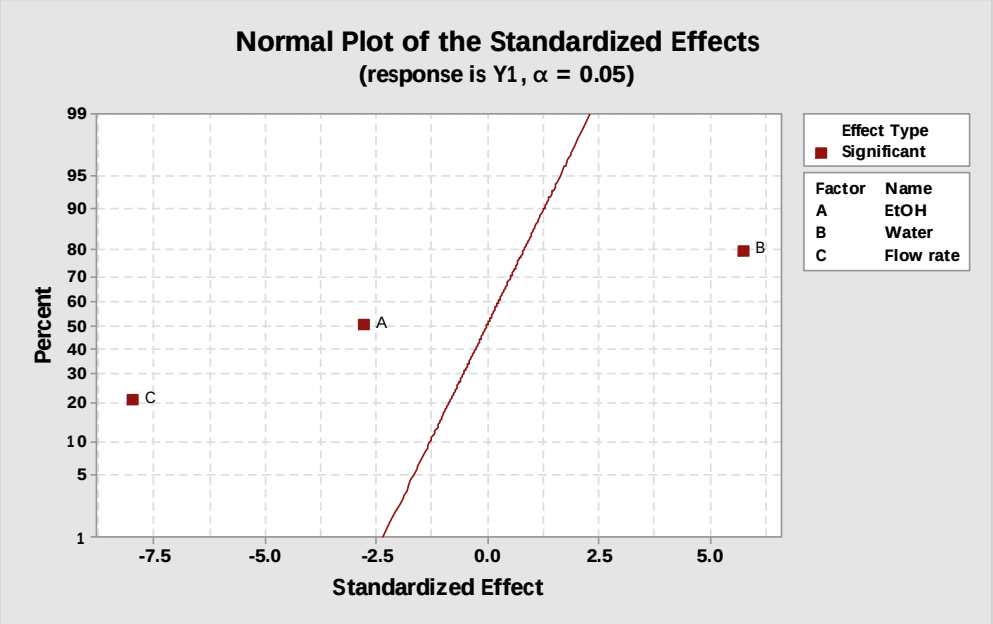


Figure (3.42) Normal plot of the standard effect for tailing factor (Y_2) for HPLC method 2 for assay of esomeprazole.

Retention time response would be increased as ratio of water increased, while it would decrease by increasing both ratio of ethanol and flow rate, as shown in Figure (3.43).

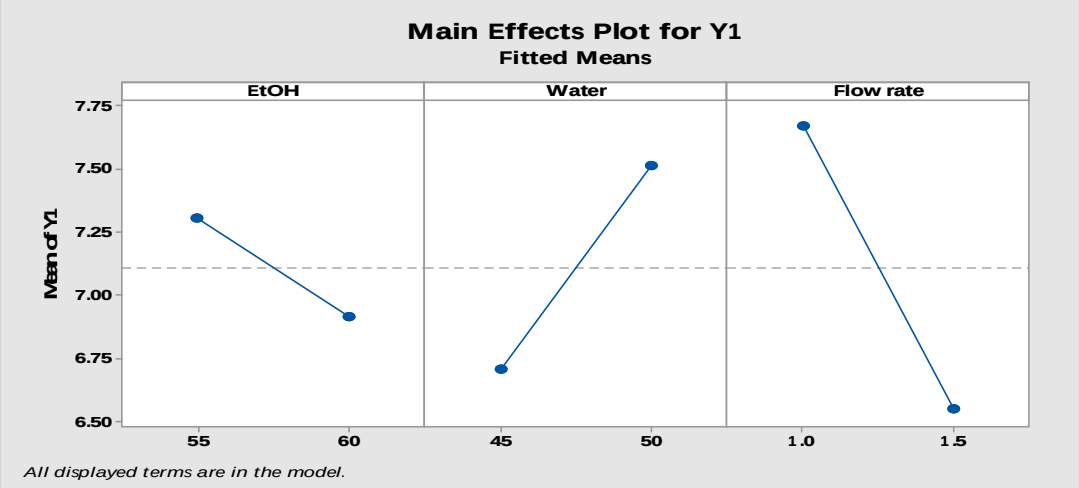


Figure (3.43) The main effect of the three factors affecting tailing factor at p value 0.05 for HPLC method 2 for assay of esomeprazole.

The recommended optimized chromatographic conditions for HPLC method 2 for assay of esomeprazole were summarized in Table (3.47).

Table (3.47) Optimum chromatographic conditions for HPLC method 2 for assay of esomeprazole.

Condition	**Optimum**
Column	Gemini Nx C_{18} (110Å, 250×4.6 mm) fully porous silica
Mobile phase composition	55:45 (EtOH:water)
Flow rate	1.5 ml/min
Detection wavelength	302 nm
Injection volume	10 µL
Temperature	ambient

3.2.2.4.2 System suitability

The result for system suitability was obtained by injecting one concentration six times, and then percentage RSD was calculated as described in Table (3.48).

Table (3.48) System suitability data for determination of esomeprazole using HPLC method 2.

Exp No.	Retention time (min)	Peak area	Tailing factor	Theoretical plates
1	6.227	819780	1.251	4714.7
2	6.239	819609	1.247	4727.1
3	6.248	817785	1.247	4729.9
4	6.247	815553	1.247	4720.6
5	6.253	817485	1.245	4742.2
6	6.253	818411	1.247	4722.9
Mean	6.245	818104	1.247	4726.2
%RSD	0.160	0.191	0.158	0.200
Limit	NMT 1%	NMT 2%	NMT 2%	NLT 2000

3.2.2.4.3 Linearity and range

Linearity of this method was found to be in range from 2.5 to 40 µg/ml as in Figure (3.44).

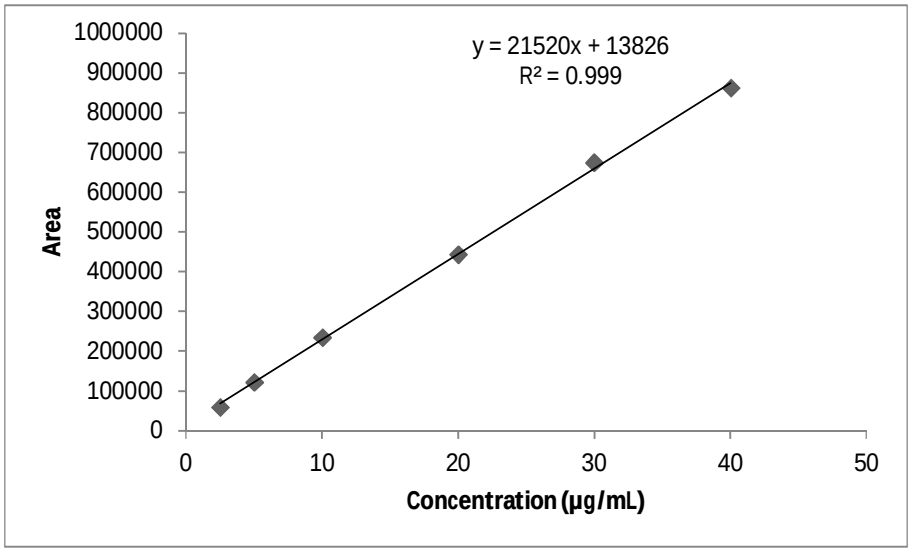


Figure (3.44) Calibration curve for determination of esomeprazole using HPLC method 2.

3.2.2.4.4 Precision

Intra-day and inter-day precision for determination of esomeprazole using HPLC method 2 were studied and results show that the method is precise according to the low values of standard deviation (SD), and percent relative standard deviation (% RSD), as shown in Table (3.49).

Table (3.49) Evaluation of intra- and inter-day precision for the propose HPLC method 2 for assay of esomeprazole.

Taken (µg/ml)	Intra – day precision			Inter – day precision			Accepted criteria
	Found (µg/ml)	SD	% RSD	Found (µg/ml)	SD	% RSD	
15	15.04	0.075	0.050	15.043	0.003	0.021	%RSD ≤ 2%
25	25..12	0.025	0.100	25.103	0.043	0.175	
25	34.79	0.038	0.109	34.796	0.025	0.071	

3.2.2.4.5 Recovery (accuracy)

Accuracy was estimated in terms of percent recovery and percent relative standard deviation. Values of t-test and F-test had been calculated using USP standard reference

HPLC method. These values were less than their permissible values of: 2.776 and 19.0 for t- and F-test, respectively, at 95% confidence level, indicating a high accuracy and precision of the method as shown in Table (3.50).

Table (3.50) Accuracy studies of the proposed HPLC method 2 for assay of esomeprazole.

Taken (µg/ml)	Found (µg/ml)	% Recovery	% RSD	Proposed Method Mean ± SD	Reference Method Mean ± SD	Accepted criteria	t-test	F-test
23.5	23.68	100.78	0.642	100.092 ± 0.643	99.97± 0.416	98 -102	0.365	16.11
26.5	26.348	99.43						
28.5	28.54	100.14						

3.2.2.4.6 Sensitivity

Sensitivity of the method was investigated by measurement of limit of detection (LOD), and limit of quantification (LOQ), which was found to be 1.172 and 3.551 µg/ml, respectively. Their values proved that the proposed method was sensitive for quantification of esomeprazole magnesium.

3.2.2.4.7 Selectivity

The method was found to be selective for esomeprazole since it gave no response for placebo solution as shown in Figure (3.45).

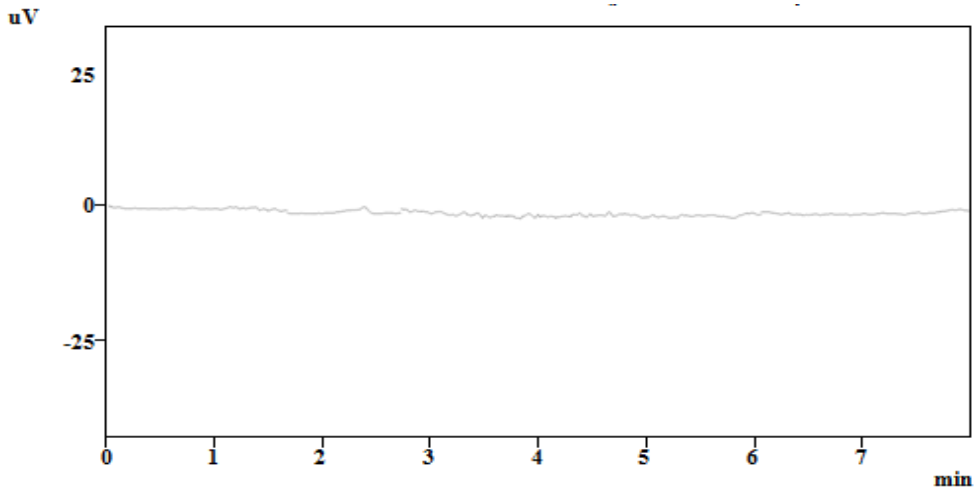


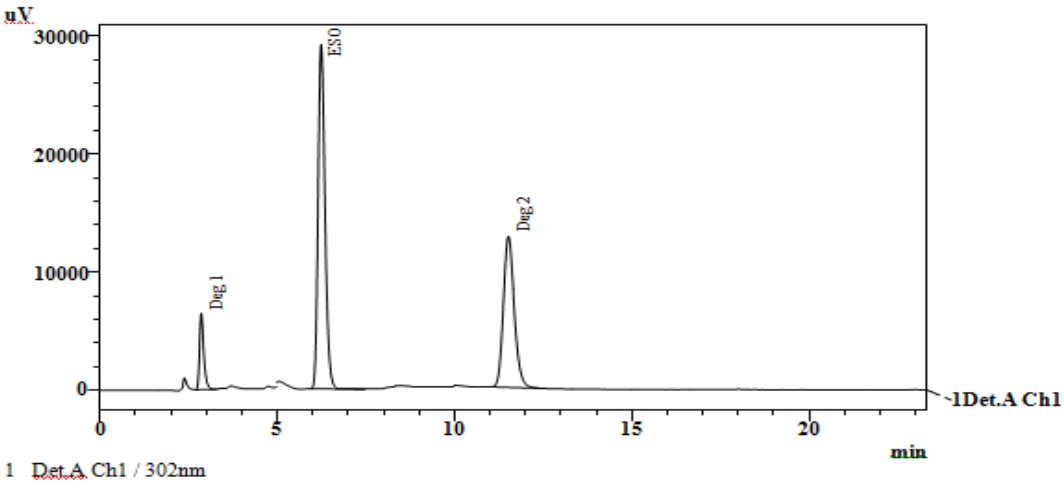
Figure (3.45) Chromatogram of placebo solution for esomeprazole using HPLC method 2.

3.2.2.4.8 Specifity

To study Specifity of the proposed method, stress degradation was performed using different stress conditions: acid, base, oxidation, thermal and photolytic as shown in Table (3.51). Under acidic stress condition, two degradation products were detected as in Figure (3.46), under oxidation stress condition, two degradation products were detected as shown in Figure (3.47), and under photolytic stress condition, two degradation products were detected as shown in Figure (3.48).

Table (3.51) Degradation study of esomeprazole under various conditions using HPLC method 2.

No.	Condition	Assay %
1	Acid	55.63
2	Base	94.10
3	Oxidation	75.80
4	Thermal	96.32

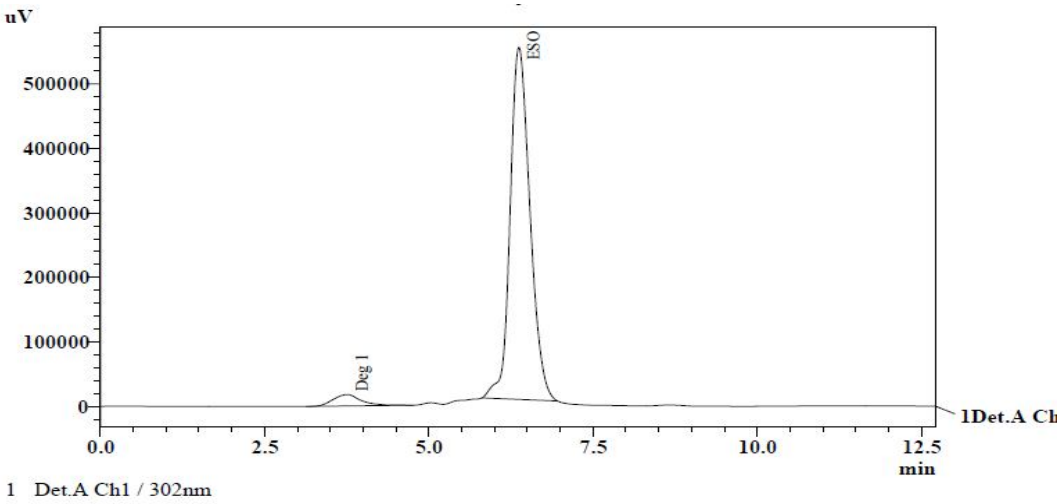


PeakTable

Detector A Ch1 302nm

Name	Ret. Time	Area	Resolution	Tailing Factor	Theoretical Plate#
Deg 1	2.855	54744	0.000	1.544	2517.843
ESO	6.239	392315	11.568	1.264	4871.836
Deg 2	11.513	273570	11.506	1.210	6783.121
		720630			

Figure (3.46) Chromatogram of acid stress degradation of esomeprazole using HPLC method 2.

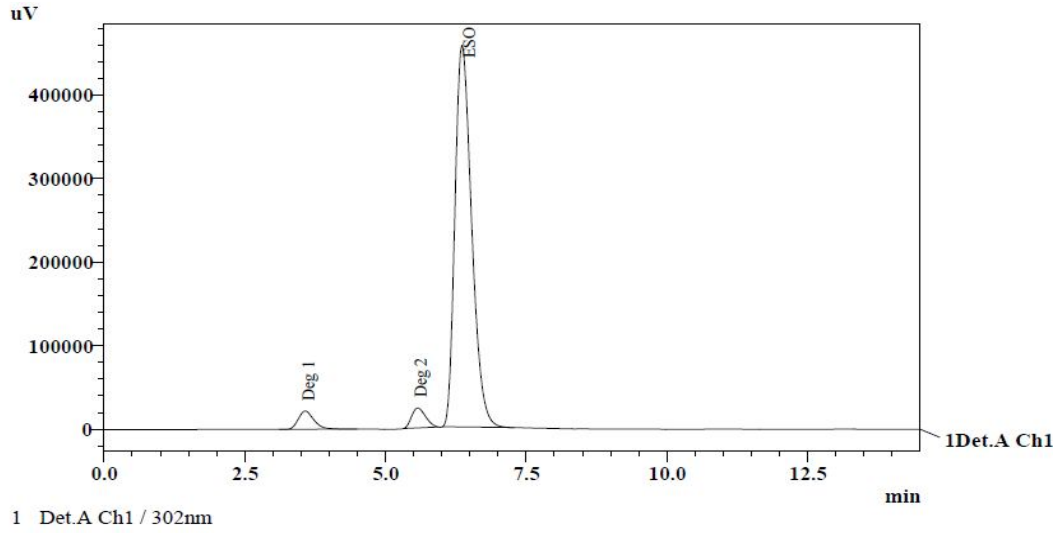


PeakTable

Detector A Ch1 302nm

Name	Ret. Time	Area	Resolution	Tailing Factor	Theoretical Plate#
Deg 1	3.757	527551	0.000	1.205	2414.974
ESO	6.368	11494976	4.064	1.190	3168.720
		12022527			

Figure (3.47) Chromatogram of oxidation stress degradation of esomeprazole using HPLC method 2.



PeakTable

Detector A Ch1 302nm

Name	Ret. Time	Area	Resolution	Tailing Factor	Theoretical Plate#
Deg 1	3.570	420487	0.000	1.326	2797.208
Deg 2	5.569	408308	4.097	1.256	3248.018
ESO	6.356	9427891	1.548	1.360	4164.317
		10256686			

Figure (3.48) Chromatogram of photo stress degradation of esomeprazole using HPLC method 2.

3.2.2.4.9 Robustness

Robustness of the method was measured by the capability of the method to remain unaffected by small variation in method parameters. For this measurement effects of changing three factors were studied: mobile phase composition, wavelength of detection and flow rate, as shown in Tables (3.52), (3.53), and (3.54).

Table (3.52) Effect of changing mobile phase composition on the performance of HPLC method 2 for the assay of esomeprazole.

Factor			Response				Accepted criteria
Mobile phase composition			R_t (min)	% RSD	Area	% RSD	%RSD ≤ 2%
Original	Level	Used					
55 : 45	+1	56 :44	6.098	0.234	572258	0.039	
	0	55 :45	6.273	0.088	588308	0.351	
	-1	54 : 46	6.827	0.062	568209	0.174	

Table (3.53) Effect of changing wavelength of detection on the performance of HPLC method 2 for the assay of esomeprazole.

Factor			Response				Accepted criteria
Wavelength of detection (nm)			R_t (min)	% RSD	Area	% RSD	%RSD ≤ 2%
Original	Level	Used					
302	+1	304	6.291	0.060	587887	0.170	
	0	302	6.273	0.088	588308	0.351	
	-1	300	6.276	0.153	580077	0.045	

Table (3.54) Effect of changing flow rate on the performance of HPLC method 2 for the assay of esomeprazole.

Factor			Response				Accepted criteria
Flow rate (ml/min)			R_t (min)	% RSD	Area	% RSD	%RSD ≤ 2%
Original	Level	Used					
1.5	+1	1.65	5.697	0.098	534973	0.007	
	0	1.5	6.273	0.088	588308	0.351	
	-1	1.35	6.973	0.093	657287	0.070	

3.3 Stability of Amlodipine besylate using HPLC method 1

3.3.1 Effect of sunlight on stability of amlodipine solution

Table (3.55) shows the effect of sunlight on amlodipine besylate. The graphical representation between time and assay according to the zero order reaction equation: [(a-x)= -kt + a] gave a straight line with negative slope and intercept, this indicate that effect of sunlight was of zero order with rate constant 7.592 area hour^{-1}, and $t_{1/2}$ of 6.82 hour.

Table (3.55) Effect of sun light on stability of amlodipine besylate solution using HPLC method 1.

Time (hour)	% Assay
1	95.66454
2	88.28537
3	82.49763
4	71.83103
5	65.92942

3.3.2 Kinetics of hydrolysis of amlodipine in basic medium at 80° C

Table (3.56) shows the effect of thermal hydrolysis on amlodipine besylate in basic medium. The graphical representation between time and assay according to the zero order reaction equation: [(a-x)= -kt + a] gave a straight line with negative slope and intercept, this indicate that effect of thermal hydrolysis was a zero order reaction with rate constant 0.730 area min^{-1}, and $t_{1/2}$ of 74.12 min.

Table (3.56) Hydrolysis of amlodipine in basic medium at 80° C using HPLC method 1.

Time (min)	% Assay
10	99.48361
20	96.00184
30	85.89624
40	79.06064
50	70.96117
60	64.7536

3.3.3 Kinetics of hydrolysis of amlodipine solution at 80° C

Table (3.57) shows the thermal degradation of amlodipine besylate at 80° C. The graphical representation between time and 1/assay according to the second order reaction equation: [1/(a-x) = kt + 1/a] gave a straight line with positive slope and intercept, this indicate that effect of thermal hydrolysis was a second order reaction with rate constant 0.02 $hour^{-1}area^{-1}$, and $t_{1/2}$ of 5000 hour.

Table (3.57) Hydrolysis of amlodipine besylate solution at 80° C using HPLC method 1.

Time (hour)	% Assay	1/c
1	94.800	0.0105
2	93.116	0.0107
3	91.527	0.0110
4	89.188	0.0112
5	87.251	0.0115

3.3.4 Effect of some excipients in hydrolysis of amlodipine at 80° C

Table (3.58) shows that all values of rate constant of thermal hydrolysis in presence of different excipients was less than that calculated without excipients as shown in section 3.3.3, indicating that their addition during formulation of amlodipine besylate increased stability of the drug as a result.

Table (3.58) Effect of excipients on hydrolysis of amlodipine besylate solution at 80° C using HPLC method 1.

Excipient	Concentration	pH	% Assay	Rate constant
Magnesium stearate	0.1 %(w/v)	8.35	87.82	9.18×10^{-5}
Sodium starch	0.1 %(w/v)	7.421	85.53	9.81×10^{-4}
Di basic calcium phosphate	0.1 %(w/v)	7.539	88.65	9.00×10^{-4}
Microcrystalline cellouse	0.1 %(w/v)	6.961	91.56	5.06×10^{-4}

3.3.5 Effect of controlled temperature 100° C on solid amlodipine

Table (3.59) shows that the effect of controlled temperature at 100° C on stability amlodipine besylate. The graphical representation between time and ln (assay) according to first order reaction equation: [ln(a-x) = -kt + ln a] gave a straight line with negative slope and intercept, this indicate that effect of controlled temperature was a first order reaction with rate constant of 0.051 day^{-1}, and $t_{1/2}$ of 13.59 day.

Table (3.59) Effect of controlled temperature 100° C on stability of amlodipine besylate using HPLC method 1.

Time (days)	% Assay	ln C
1	97.77	4.58
2	93.55	4.54
3	89.36	4.49
4	83.71	4.43

Amlodipine besylate stability was affected by hydrolysis, oxidation, heat, and sun light. Hydrolysis of amlodipine took place due to presence of two ester groups; the rate of hydrolysis was affected by heat and presence of bases. Presence of bases increased the rate of hydrolysis more than heating because the half time of hydrolysis in presence of bases was less than that when heating as shown in section 3.3.2 and 3.3.3.

Oxidation of amlodipine was due to presence of amine groups (both primary and secondary), while the effect of sun light might be due to dehydrogenation of amine group in the ring.

According to the stability study amlodipine besylate was almost affected by all factors affecting stability; so when manufacturing this drug alone or in combination with other drugs special care might be taken to prevent its degradation.

3.4 Stability of esomeprazole magnesium HPLC method 1

3.4.1 Effect of sunlight on stability of esomeprazole solution

Table (3.60) shows that the effect of sun light on esomeprazole. The graphical representation between time and ln (assay) according to the first order reaction equation: [ln(a-x) = -kt + ln a] gave a straight line with negative slope and intercept, this indicate that effect of was a first order with rate constant of 0.019 min^{-1}, and $t_{1/2}$ of 36.48 min.

Table (3.60) Effect of sunlight on stability of esomeprazole solution using HPLC method 1.

Time (min)	% Assay	ln C
10	97.83	0.0102
20	89.47	0.0112
30	70.67	0.0142
40	59.80	0.0167
50	48.76	0.0205
60	36.92	0.0271

3.4.2 Kinetics of thermal degradation of esomeprazole in acidic medium at room temperature

Table (3.61) shows that acid degradationtion of esomeprazole at 80° C. The graphical representation between time and ln (assay) according to the first order reaction equation: [ln(a-x) = -kt + ln a] gave a straight line with negative slope and intercept, this indicate that effect of thermal degradationtion in acidic medium was a first order reaction with rate constant of 0.046 min^{-1}, and $t_{1/2}$ of 15.07 min.

Table (3.61) Acid degradation of esomeprazole solution at room temperature using HPLC method 1.

Time (min)	% Assay	ln C
5	97.58	4.581
15	70.48	4.255
25	45.36	3.815
35	27.00	3.296
45	15.56	2.745

3.4.3Kinetics of thermal degradation of esomeprazole solution at 80° C

Table (3.62) shows that thermal degradation of esomeprazole at 80° C. The graphical representation between time and assay according to the zero order reaction equation:

[(a-x)= -kt + a]gave a straight line with negative slope and intercept, this indicate that effect of thermal degradationtion was a zero order reaction with rate constant of 7.363 area hour^{-1}, and $t_{1/2}$ of 6.01 hour.

Table (3.62) Degradationtion of esomeprazole solution at 80° C using HPLC method 1.

Time (hour)	% Assay
1	94.94
2	85.55
3	77.27
4	67.27
5	61.31

3.4.4 Effect of some excipients in thermal degradation of esomeprazole at 80° C

Comparison of the values of rate constants of thermal hydrolysis when excipients were dded with those calculated without addition of excipients as in section 3.4.3 indicated that addition of excipients increased the stability of esomeprazole as shown in Table (3.63).

Table (3.63) effect of excipients on degradationtion of esomeprazole solution at 80° C using HPLC method 1.

Excipient	Concentration	pH	% Assay	Rate constant
Magnesium stearate	0.1 %(w/v)	10.08	84.41	3.279
Starch maize	0.1 %(w/v)	10.10	82.11	5.714
Talcum powder	0.1 %(w/v)	9.92	87.20	4.863
Microcrystalline cellouse	0.1 %(w/v)	10.03	85.08	3.341

3.4.5 Effect of controlled temperature 100° C on solid esomeprazole

Table (3.64) shows that the effect of controlled temperature at 100° C on stability of esomeprazole. The graphical representation between time and assay according to the zero order reaction equation: [(a-x)= -kt + a] gave a straight line with negative slope and

intercept, this indicate that effect of heat was a zero order reaction with rate constant of 7.853 area day^{-1}, and $t_{1/2}$ of 6.51 days.

Table (3.64) Effect of controlled temperature 100° C on stability of esomeprazole using HPLC method 1.

Time (days)	% Assay
1	91.61
2	89.26
3	81.64
4	67.98

Esomeprazole stability was affected by several factors including degradationtion, oxidation, UV light at 254 nm, heat, and sun light. Rate of hydrolysis was affected by both heat and presence of acids, but presence of acids increases the rate of hydrolysis more than heat. Its oxidation was caused by the presence of secondary amine functional group. Effect of sun light and photo degradation might be due to dehydrogenation of amine group.

According to this stability study it is revealed that stability of esomeprazole magnesium was affected by all factors affecting stability, especially hydrolysis in acidic medium and exposure to sun light; so special care must be taken during manufacturing and storage of this drug alone or in combination with other drugs.

3.5 Conclusion and recommendations

It can be concluded and/or recommended from this work that:

- UV spectra of amlodipine besylate and esomeprazole magnesium in different solvents depend mainly on polarity of solvent used.
- New spectrophotometric methods in the UV as well as VIS region were developed for the determination of amlodipine besylate and esomeprazole magnesium in pure and pharmaceutical formulations. The developed methods were based either on solubility enhancement using hydrotropic techniques or derivatiztion of both drugs using either ion-pair formation reactions with methyl orange and eriochrome black T dyes or miscellaneous methods using alizarin red

s. These developed methods were fully validated according to ICH guidelines and found to be sensitive, precise and accurate.

- These methods utilized simple, inexpensive and widely available UV-VIS spectrophotometers.
- New stability indicating RP_HPLC methods were also developed for determination of both drugs in pure and pharmaceutical dosage forms in presence of their degradation products. These methods were optimized using factorial design approach which saves time and effort, reduces also the consumption of reagents and thus reduces the cost. These methods were fully validated for linearity, selectivity, precision, accuracy, robustness, and specifity. The developed methods were isocratic and utilized different columns (C_{18} and C_{12}) and different organic solvents including acetonitrile, methanol, and ethanol in different ratios with water.
- The develop methods were suitable for studying stability of both drugs in different conditions, and were capable of detecting some of degradation products.
- Both drugs were found to be affected with almost all factors affecting stability of drugs, but esomeprazole was found to be more affected.
- Study of stability of amlodipine besylate revealed that its stability mainly affected by sunlight and hydrolysis. The rate of hydrolysis was increased by both heating and addition of bases.
- On the other hand stability of esomeprazole magnesium was mainly affected by exposure to sunlight and hydrolysis. The rate of hydrolysis increased by both heating ad addition of acids.
- For both drugs addition of excepients was found to increase the stability of the drug toward hydrolysis.
- According to the results of stability study special care must be taken during formulation and storage of both drugs, since their stability was sensitive toward heat and exposure to sunlight.
- UV/VIS methods were recommended to be used for analysis of pure API, while RP-HPLC methods could be used in both pure and pharmaceutical formulations even in presence of degradation products.

- Hyphenated techniques like LC/MS, LC/MS/MS, and LC/NMR were recommended to be used in stability indicating methods for detection and identification of degradation products.
- Also long term stability studies for both drugs were recommended to assess the effect of humidity and combine heat/humidity effect for long time intervals.

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