1. INTRODUCTION

1.1 Stability of Drugs

Stability testing forms an integral part of formulation development. It is important to assess the effect of temperature and humidity on stability of drug and dissolution drug release rate. It helps to generate information for predicting the shelf life of the drug and recommended storage conditions.

Chemical stability of pharmaceutical molecules is a matter of great concern as it affects the safety and efficacy of the drug product. The Food and Drug Administration (FDA) and International conference of harmonization (ICH) guidance's state the requirement of stability testing data to understand how the quality of a drug substance and drug product changes with time under the influence of various environmental factors .Knowledge of the stability of molecule helps in selecting proper formulation and package as well as providing proper storage conditions and shelf life, which is essential for regulatory documentation. Forced degradation is a process that involves degradation of drug products and drug substances at conditions more severe than accelerated conditions and thus generates degradation products that can be studied to determine the stability of the molecule.

The ICH guideline states that stress testing is intended to identify the likely degradation products which further helps in determination of the intrinsic stability of the molecule and establishing degradation pathways, and to validate the stability indicating procedures used (Blessy *et al.*, 2014).

Pharmaceutical analysis provides information on the identity, purity, content and stability of starting material, excipients and active pharmaceutical ingredients (APIs). A distinction is made between analysis of the pure active ingredients and pharmaceutical formulations.

Specification and test methods for the commonly used API and excipients are described in detail in pharmacopoeias.

Stability indicating methods are quantitative test methods that can detect changes of API and drug products during time and under certain conditions. Information on type and amount of degradation products over time is important for quality, safety and efficacy of the drug. Therefore, Food and Drug Administration (FDA), European Medicines Agency and other regulatory agencies, along ICH and good manufacturing practice require development and validation of stability indicating methods. General purpose of stability testing is to provide evidence on how the quality of an API or a finished pharmaceutical product changes during time under the influence of different environmental factors such as temperature, humidity and light. After these tests have been performed, recommendation on storage conditions and shelf life of the product can be given. ICH guidelines give detailed description of forced decomposition studies (stress testing). Chemical stability of pharmaceutical molecules is a matter of great concern as it affects the safety and efficacy of the drug product. But these guidelines are very general in conduct of forced degradation and do not provide details about the practical approach towards stress testing. Although forced degradation studies are a regulatory requirement and scientific necessity during drug development, it is not considered as a requirement for formal stability program, (Klick *et al*, 2005), (Alsante and Baertchi, 2003).

Pharmaceuticals should at all times be stored under the recommended conditions, as stated by the manufacturing company, thus preventing contamination and deterioration of the product (BP, 2009). Medications may lose their potency and become less effective if stored inappropriately. Good storage ensures that medications are safe for their intended use, without causing adverse drug reactions. Drug stability means the ability of the pharmaceutical dosage form to maintain the physical, chemical, therapeutic and microbial properties during the time of storage and usage by the patient.

1.2 Accelerated Stability Studies

Storage condition of 40 ^{0}C and relative humidity of 75% RH has been recommended for all the four zones for drug substances and drug products. Accelerated storage conditions must be at least 15 ^{0}C above the expected actual storage temperature and appropriate relative humidity, table (1.1), (ICH, 2003).

Table (1.1) Accelerated and Intermediate Testing Conditions

Climatic Zone	Temperature	Humidity	Minimum Duration
Accelerated Ambient	40ºC ± 2ºC	75% ± 5% RH	6 Months
Accelerated Refrigerated	25ºC ± 2ºC	60% ± 5% RH	6 Months
Accelerated Frozen	5ºC ± 3ºC	No Humidity	6 Months
Intermediate	30ºC ± 2ºC	65% ± 5% RH	6 Months

Information about the stability of the drug substance (new chemical entity) or preliminary drug product formulations are an integral part of the systematic approach towards stability evaluation.

Accelerated stability studies are performed to establish at short notice what the intrinsic stability of drug substance or formulation is, and to help to predict what can be expected during the formal long term stability study.

1.3. Stress testing

Stress testing degradation is a degradation of new drug substance and drug product at conditions more severe than accelerated conditions. It is required to demonstrate specificity of stability indicating methods and also provides an insight into degradation pathways and degradation products of the drug substance and helps in elucidation of the structure of the degradation products. Forced degradation studies show the chemical behavior of the molecule which in turn helps in the development of formulation and package (ICH, 2003).

Stress testing of the API can help to identify possible degradation products. It should include the effect of temperature (in 10 ^{0}C increments), humidity (≥75% relative humidity), oxidation, photolysis and hydrolysis of the API at a wide range of pH (acidic, neutral and alkali conditions).

Stress testing degradation studies are carried out to achieve the following purposes:

Establish degradation pathways of drug substances and drug products, differentiate degradation products that are related to drug products from those that are generated from non-drug product in a formulation., elucidate the structure of degradation products, determine the

intrinsic stability of a drug substance in formulation, reveal the degradation mechanisms such as hydrolysis, oxidation, thermolysis or photolysis of the drug substance and drug product, establish stability indicating nature of a developed method, understand the chemical properties of drug molecules, generate more stable formulations, produce a degradation profile similar to that of what would be observed in a formal stability study under ICH conditions and solve stability-related problems. (Martindale, 2006)

1.4. Method development considerations

There are many factors to consider when developing methods. The initial steps include collecting as much information about the analyte in regard to the physicochemical properties (pK_a, log P, solubility) and determining which mode of detection would be suitable for analysis (i.e., suitable wavelength in case of UV detection). Sample preparation, which includes centrifugation, filtration, and/or sonication and type of diluent, plays an integral role in method development because this may affect the chromatography and the recovery of the analytes. Determination of the solution stability in the diluent is also important during early method development. If the solution is not stable, it will become increasingly more challenging to compare subsequent method development analysis. Choice of the mobile-phase and gradient conditions is dependent on the nature of the analyte and the hydrophobicity of the analytes in the mixture respectively.

1.4.1 High Performance Liquid chromatography (HPLC)

HPLC is currently the most widely used method of quantitative analysis in the pharmaceutical industry and in pharmaceutical analysis laboratories. The most important thing in HPLC is to obtain the optimum

resolution in the minimum time. A resolution value of 1.5 or greater between two peaks will ensure that the sample components are well (baseline) separated to a degree at which the area or height of each peak may be accurately measured.

The quantification methods incorporated in HPLC are borrowed mostly from gas chromatography methods. The basic theory for quantitation involves the measurement of peak height or peak area. To determine the concentration of a compound, the peak area or height is plotted versus the concentration of the substance. For peaks that are well resolved, both peak height and area are proportional to the concentration. . (Moffat *et al.*, 2004)

1.4. 2 Detector consideration

Choice of the proper detection scheme is dependent on the properties of the analyte. Different types of detectors are available such as ultraviolet (UV), fluorescence, electrochemical, light scattering, refractive index (RI), flame ionization detection (FID), evaporative light scattering 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 UV detection

1.4.3. Choice of stationary phase

Ideally for reverse- phase separation, the retention factors or capacity factor (k) for all components in the sample should lie between 1 and 10 to achieve separation in a reasonable time., for a given stationary phase the k of particular component can be controlled by changing the solvent composition of the mobile phase; however, the impact of eluent composition will depend on the type of stationary phase and the nature of components in the mixture.

In reversed-phase HPLC the most common solvent mixtures are: water and acetonitrile, water methanol, and water and Tetrahydrofuran (THF).The elution increases as the organic portion of the modifier increases. Thus, to optimize a chromatographic separation, the concentration of the organic modifier is adjusted so that the capacity factor (k) of the components in the sample are in the range of 1 to 10, however, due to the hydrophobic nature of the compound, even high concentrations of organic modifier will not allow elution of all components in a single run and the chromatographer can try one or combination of the following approaches: use a stronger modifier, apply a steeper gradient , use less hydrophobic stationary phase and the type of column chosen for a particular separation depends on the compound and the aim of analysis. C_8 or C_{18} which stable at pH<2, pH 2-8.

1.4.4 Mobile phase consideration

1.4.4.1 Choice of pH.

If analytes are ionizable, the proper mobile phase pH must be chosen based on the analyte pKa.

1.4.4.2 Buffer

In order to develop rugged HPLC methods, knowledge of choosing the right buffer is very important. Buffers that are selected should have a good buffering capacity at specific mobile-phase pH., the concentration of the buffer should be at least 10 m mole to provide the needed ionic strength to suppress any undesired analyte solvation effect that may lead to poor peak shapes.

1.4.4.3 Choice of organic modifier.

Selection of the organic modifier type could be viewed as relatively simple: the usual choice is between acetonitrile and methanol (rarely THF), acetonitrile as organic modifier may offer these variations due to the introduction of a dual retention mechanism. The viscosity of water/organic mixture should be considered as additional parameter in the selection of organic modifier. Acetonitrile/water mixtures show lower viscosity than equivalent methanol/water mixtures, acetonitrile 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, (Chandrul and Srivatava, 2010).

In reverse phase, the column size is the same, but silica is modified and made non-polar by attaching long hydrocarbon chains to its surface - typically with either 8 or 18 carbon atoms in them. A polar solvent, for example, a mixture of an alcohol such as methanol and water is used. There will be a strong attraction between the polar solvent and polar molecules in the mixture being passed through the column. The attraction between the hydrocarbon chains attached to the silica (the stationary phase) and the polar molecules in the solution won't be as much. Therefore, polar molecules in the mixture will spend most of their time moving with the solvent. Non-polar compounds in the mixture will tend to form attractions with the hydrocarbon groups because of Van-der Waals forces. They will be less soluble in the solvent because of the need to break hydrogen bonds as they squeeze in between the water or methanol molecules. They spend less time in solution in the solvent and this will slow them down on their way through the column. This means that now it is the polar molecules that will travel through the column more quickly. Reversed phase HPLC is the most commonly used form of HPLC. The sample to be analyzed is injected

in a small volume into the stream of the mobile phase. The motion of analyte through the column is slowed by specific chemical or physical interactions with the stationary phases as it traverses the length of the column. The amount the analyte is slowed depends on the nature of the analyte and on the compositions of the stationary and mobile phases. Time taken by a specific analyte to elute is called retention time; the retention time under particular conditions is considered a reasonably unique identifying characteristic of a given analyte. Smaller particle size column packing (which creates a higher back-pressure) increases the linear velocity giving the components less time to diffuse within the column, which leads to improved resolution in the resulting chromatogram. Commonly used solvents include any miscible combination of water or various organic liquids (most common being methanol and acetonitrile). Water may contain buffers or salts to help in separation of the analyte components or compounds such as trifluoroacetic acid which acts as an ion pairing agent. A further refinement to HPLC has been to change the mobile phase composition during the analysis, this is known as gradient elution, (Joseph, 2008).

A general gradient for reversed phase chromatograph might start at 5% methanol and progresses gradually to 50% methanol over 25 minutes; the gradient chosen depends on the hydrophobicity of the analyte. The analyte mixtures are separated as a function of the affinity of the analyte for the current mobile phase composition relative to the stationary phase. This process of portioning is similar to that which occurs during a Liquid-liquid extraction but this is continuous and not step-wise. For example, when using a low water/ high methanol gradient, the more hydrophobic

components will elute from the column due to a relatively hydrophobic mobile phase. The hydrophilic compounds will elute under conditions of relatively low methanol/high water. The choice of solvents, additives and gradient depend on the nature of the analyte and the stationary phase, generally a series of tests are performed on the analyte and a number of trial runs may be processed in order to find the optimum HPLC method giving the best separation of peaks (Snyder *et al.*, 2011).

1.5 Infrared spectroscopy

IR spectroscopy has a great potential in elucidating the molecular structure, infrared spectrum of an organic substance is like a fingerprint and allows to identify a substance by the comparison with previously recorded spectra or to put in evidence important structural parts of the molecules with intense vibration bands, even if the substance is in mixture or complexes.

Fourier transform infrared spectroscopy is a technique based on the determination of the interaction between an IR radiation and a sample that can be solid, liquid or gaseous. It measures the frequencies at which the sample absorbs, and also the intensities of these absorptions. The frequencies are helpful for the identification of the sample's chemical make-up due to the fact that chemical functional groups are responsible for the absorption of radiation at different frequencies. The concentration of component can be determined based on the intensity of the absorption. The spectrum is a two-dimensional plot in which the axes are represented by intensity and frequency of sample absorption (Raphael, 2011).

1.6 UV-VIS spectrophotometer

It is an absorption spectroscopy in which the excitation of electrons is accompanied by change in vibrational and rotational quantum number, so that an absorption line becomes a broad peak containing vibrational rotational fine structure. Due to interaction of solute with solvent molecules, this is usually clear out and smooth curve is observed. The UV region extends from 10 to 380 nm, but the most analytically useful region is from 200 to 380 nm, called as near UV region. Below 200 nm, the air absorbs appreciably and so the instruments operated under a vacuum, hence this wavelength region is called the vacuum UV. The visible region is actually a very small part of the electromagnetic spectrum and it is the region of wavelength, which can be observed by the human eye, where light, appears as a color. The visible region extends from 380 nm to about 780 nm.

The factors that influencing the absorption of radiant energy, an absorbing group, solvent effect, effect of temperature, inorganic ions and Absorbing group or chromophores. A chromophore is a group which attached to a saturated hydrocarbon produces a molecule that absorbs a maximal of visible of UV energy at some specific wavelength, absorption band of many substances are relatively sharper and may also exhibit fine structure when measured in solvent of low dipole moment. Interactions of solvent solute are found to be much stronger in each substances where strong dipole forces are involved. The solvent effects do help in recognizing electronic transitions of the type n —›π that essentially involve the non-bonding electrons of nitrogen and oxygen.

The spectrum normally shows appreciable changes with varying pH when an ioniziable moiety is present in the molecule and thereby

constituents part of chromophore structure. Low temperature offer sharper absorption bands of many pharmaceutical substances than at room temperature due to fewer vibrational levels are occupied and the degree of solute – solvent interaction is minimized. It is worth to note that the absorption spectra for inorganic ions elements are caused due to a charge transfer- process whereby an electron gets transferred from one part of the ion to another (Prachi, 2011). Ultraviolet-visible spectrophotometry is one of the most frequently employed technique in pharmaceutical analysis. It involves measuring the amount of ultraviolet or visible radiation absorbed by a substance in solution. Derivative spectroscopy uses first or upper derivatives of absorbance with respect to wavelength for qualitative investigation and estimation. Derivative spectra can be used to enhance differences among spectra, to resolve overlapping bands in qualitative analysis and, most importantly, to reduce the effects of interference from scattering, matrix, or other absorbing compounds in quantitative analysis, (Davidson, 2002).

1.7 Method Validation

Analytical methods validation is an important regulatory requirement in pharmaceutical analysis. Method validation provides documented evidence, and a high degree of assurance that an analytical method employed for a specific test is suitable for its intended use.

Validation is the process to confirm that the analytical procedure employed for specific test is suitable for intended use and that they support, the identity, quality, purity and potency of the drug substances and drug product., method development and validation has direct impact on the quality of these data, to trust the method.

1.7.1 Parameters to be evaluated

The parameters that must be evaluated include specificity, force degradation study, limit of detection LOD, limit of quantitation LOQ, linearity, precision, system precision, method repeatability, intermediate precision, method reproducibility, accuracy, recovery, solution stability and system suitability.

The USP recognizes four method categories and defines the analytical performance characteristics that must be measured to validate each method type (USP, 2002) table (1.2).

Table (1.2) Data elements required for assay validation (as per USP)

Analytical performance characteristic	Category I	Category II		Category III	Category IV
		Quantitative	Limit test		
Accuracy	Yes	Yes	*	*	No
Precision	Yes	Yes	No	Yes	No
Specificity	Yes	Yes	Yes	*	No
Detection limit	No	No	Yes	*	No
Quantitation limit	No	Yes	No	*	No
Linearity	Yes	Yes	No	*	No
Range	Yes	Yes	*	*	No

*****May be required, depending on the nature of the specific test

Category I: Analytical methods for the quantification of major components of bulk drug substances or active ingredients (including preservatives) in finished pharmaceutical products.

Category II: Analytical methods for the determination of impurities in bulk drug substances or degradation compounds.

Category III: Analytical methods for the determination of performance characteristics.

Category IV: Identification tests

1.8.2 Method validation parameters

1.8.2.1 Specificity

Specificity of analytical method is its ability to measure accurately an analyte in the presence of interference, such as synthetic precursors, excipients, enantiomers and known degradation product that may be expected in the sample matrix.

1.8.2.2 Force degradation studies

Force degradation or stress testing is undertaken to demonstrate specificity when developing stability -indicating methods.

Stability –indicating method is one that accurately quantities the active ingredient without interference from degradation product, process impurities, excipients or other potential impurities, hence, the force degradation studies should mimic the condition to which the drug substance/drug product is actually exposed during its shelf life.

A simple logic behind these studies is, ability to separate the analyte of interest from its degradation impurities formed, if any caused by quality of chemical used for manufacturing, processing, packaging, storage, shipment, and /or any unexpected exposure during shelf life of the drug substance/drug product, (WHO,2003).

1.8.2.3 Limit of detection and limit of quantitation.

LOD: Lowest amount of analyte in sample which can be detected but not necessarily quantitated, under the stated experimental condition.

LOQ: Lowest amount of analyte in sample which can be quantitatively determined with suitable precision and accuracy.

Several approaches are given in ICH guidelines for estimating LOD and LOQ depending on the procedure: instrumental /non instrumental table (1.3).

Table (1.3) LOD and LOQ approaches

Approach	LOD	LOQ
Visual inspection	Minimum level detectable	Minimum level quantifiable
Signal- to -noise ratio	2:1 or 3:1	10:1
SD of response (σ) and slope(s)	[3.3x σ]/s	[10. x σ]/s
RSD criteria	Concentration at which RSD 10 to 33%	Concentration at which RSD ≤10%

The acceptance criteria of RSD of six replicate injection is ≤ 10% for LOQ and between>10 and ≤33%. For LOD.

Others method validation acceptance criteria are shown in table (1.4)

1.8.2.4 Linearity

The linearity of an analytical procedure is its ability (within a given range) to elicit or obtain test results that are directly proportional to the concentration (amount) of analyte in the sample.

1.8.2.5 Range

The range is the interval between the upper and lower level (including these level) that have been demonstrated to be determined with precision, accuracy and linearity

Table (1.4) Method validation acceptance criteria

Validation elements	Recommendation	Acceptance Criteria
Linearity and range	Minimum 5 points 20-150% Slope Y Intercept Correlation coefficient	RSD ≤2 Steep Y close to zero $R^2 \geq 0.99$
Precision repeatability	6 replicate analysis of sample at 100% test condition	RSD ≤2
Accuracy	Triplicate spiking of placebo at 80,100 & 120% of Label Claim	98-102%
Intermediate precision	6 replicate analysis by different analyst on a different day	RSD ≤2
Specificity	Placebo Analysis Testing with known impurities Heat Stress Acid Hydrolysis Base Hydrolysis Oxidation UV	No interference

1.8.2.6 Precision

Precision is the closeness of agreement (degree of scatter) between a series of measurement obtained from multiple sampling of the same homogenous sample under the prescribed condition.

Precision may be considered at three level, system precision (system suitability), method repeatability and intermediate precision table (1.5).

Table (1.5) Relative standard deviation of precision %RSD

Procedure	System precision		Method precision		Ruggedness	
	API	DP	API	DP	API	DP
Assay	1.0/2.0	1.0/2.0	1.0	2.0	1.0	3.0
Dissolution	NA	1.0/2.0	NA	6.0	NA	5.0
Impurities	5	10	10	10	10	15

Note: NA- No applicable. API- Active pharmaceutical ingredient and DP- Drug product.

1.8.2.7 Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as conventional true value or an accepted reference value and the value found.

The logic behind this performance characteristic is whether the procedure is capable of estimation a true value or not.

1.8.2.8 Stability of system

It's often essential that solution (standard, test sample) be stable enough to allow for delays covering instrument break down / overnight analysis.

Many solutes readily decompose prior to chromatographic investigations, for example, during the preparation of the sample solutions, extraction, cleanup, phase transfer or storage of prepared vials (in refrigerators or in an automatic sampler). Under these circumstances, method development should investigate the stability of the analytes and standards. The term system stability has been defined as the stability of the samples being analyzed in a sample solution. It is a measure of the bias in

assay results generated during a preselected time interval, for example, every hour up to 46 hours, using a single solution. System stability should be determined by replicate analysis of the sample solution. System stability is considered appropriate when the RSD, calculated on the assay results obtained at different time intervals, does not exceed more than 20 percent of the corresponding value of the system precision. If, on plotting the assay results as a function of time, the value is higher, the maximum duration of the usability of the sample solution can be calculated (ICH,2005).

1.8.2.9 Robustness

Measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides indication of its reliability during its normal range. If the influence of the parameter is within a previously to tolerance, then the parameter is said to be within the method's robustness range. Typical variation include under validation programme., 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, (Robert, 2003).

1.9. Atorvastatin Calcium

Atorvastatin calcium is chemically (3R, 5R)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-propan-2-ylpyrrol-1-yl]-3, 5-dihydroxyheptanoate figure (1.1).

Atorvastatin calcium is highly susceptible to heat, moisture, low pH environment and light, also in acidic environment it degrades into corresponding lactone (Sachin *et al.*,2010) ,it has melting point 263 - 265.0 ^{0}C, pKa 4.33 and Log p about 5.7 and specific optical rotation -7 - 9.0 (Roulean, 2005).

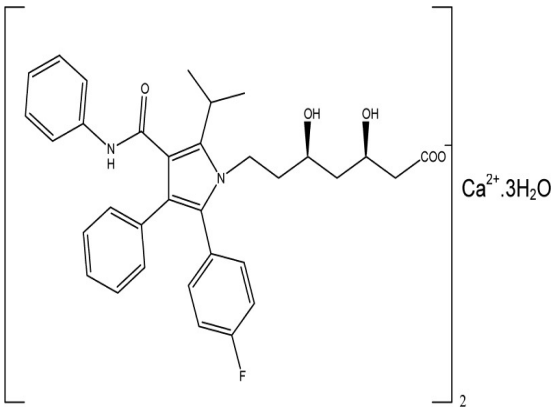


Figure (1.1) Chemical structure of Atorvastatin calcium

1.9.1 Pharmacodynamics

Atorvastatin, a selective, competitive HMG-CoA reductase inhibitor, is used to lower serum total and LDL cholesterol, and triglyceride levels while increasing HDL cholesterol, high LDL-C, low HDL-C and high TG concentrations in the plasma are associated with increased risk of atherosclerosis and cardiovascular disease. The total cholesterol to HDL-C ratio is a strong predictor of coronary artery disease and high ratios are associated with higher risk of disease. Increased levels of HDL-C are associated with lower cardiovascular risk by decreasing LDL-C and TG

and increasing HDL-C, atorvastatin reduces the risk of cardiovascular morbidity and mortality. Atorvastatin has a unique structure, long half-life, and hepatic selectivity, explaining its greater LDL-lowering potency compared to other HMG-CoA reductase inhibitors, (Conde *et al.*, 1999), (Lea, 1997) and (Ness*et al.*, 1998)

1.9.2 Pharmacokinetics and Drug Metabolism

1.9.2.1 Absorption

Atorvastatin is rapidly absorbed after oral administration with maximum plasma concentrations achieved in 1 to 2 hours. The absolute bioavailability of atorvastatin (parent drug) is approximately 14% and the systemic availability of HMG-CoA reductase inhibitory activity is approximately 30%. The low systemic bioavailability is due to presystemic clearance by gastrointestinal mucosa and first-pass metabolism in the liver.

1.9.2.2 Metabolism

Atorvastatin is extensively metabolized to ortho- and parahydroxylated derivatives and various beta-oxidation products. In vitro inhibition of HMG-CoA reductase by ortho- and parahydroxylated metabolites is equivalent to that of atorvastatin, (Conde *et al*, 1999).

1.9.2.3 Excretion

Atorvastatin and its metabolites are eliminated primarily in bile following hepatic and/or extra-hepatic metabolism; however, the drug does not appear to undergo enter hepatic recirculation. Mean plasma elimination half-life of atorvastatin in humans is approximately 14 hours, but the half-life of inhibitory activity for HMG-CoA reductase is 20 to 30 hours due to the contribution of active metabolites. Less than 2% of a dose of

atorvastatin is recovered in urine following oral administration. Statin act by competitively inhibiting HMG-Co a redactase, statins are similar to HMG-Co A at the molecular level. They bind to enzyme; making it unavailable for the substrate as a result mevalonate is not formed. (Kemketl, 2008).

1.9 .3 Methods of analysis of atorvastatin calcium

Literature survey revealed that various analytical methods such as extractive spectrophotometry, HPLC, GC-MS, LC-MS, LC electrospray tandem mass spectrometry and HPTLC method have been reported for estimation of Atorvastatin calcium from its formulations and biological fluid. (Moynul *et al*., 2011).

United States pharmacopoeia USP (2014) mentioned the determination method for impurities of Atorvastatin calcium using gradient HPLC system, the excess analysis time 32 min may limit application to routine analysis. No official analytical monograph for estimation atorvastatin calcium in IP Indian pharmacopoeia, BP.

1.10 Losartan Potassium

Losartan potassium is chemically: 2-butyl-4-chloro-1-[[2'-(1H-tetrazol-5-yl) [1, 1'-biphenyl]-4-yl] methyl]-1H-imidazole-5-methanol monopotassium salt figure (1.2) **.**Losartan is a phenyl tetrazole substituted imidazole compound which acts as a selective, angiotensin II receptor type I antagonist and is employed in the management of essential hypertension

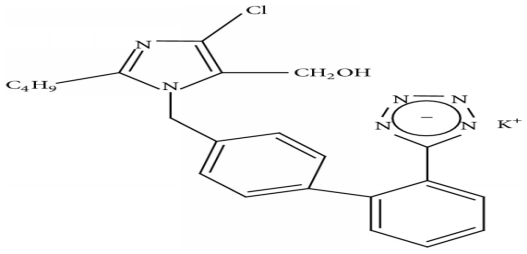


Figure (1.2) chemical structure of Losartan potassium

Losartan potassium is a white to off-white powder, which is freely soluble in water and soluble in methanol. Losartan potassium is slightly hygroscopic. It was stated that the amorphous form of losartan potassium is routinely synthesized

It has imidazole ring cyclic which is highly soluble in water, tetrazole, and benzene rings as part of its structure. It is anionic at neutral pH, which does not have (physiologically) relevant ionizable group (acidic or basic). In general, tetrazole groups are often used as metabolism-resistant isosteric replacement for carboxylic acids in a living body. Tetrazole is thermally stable group. Freely soluble in water; soluble in alcohols; slightly soluble in common organic solvents, such as acetonitrile and methyl ethyl ketone. Ionization constant pKa 5-6 (Neil, 2001).

1.10.1 Pharmacodynamics

Losartan inhibits effect of angiotensin II. A dose of 100 mg inhibits this effect by about 85% at peak highest, with 25-40% inhibition persisting for 24 hours, removal of the negative feedback of angiotensin II causes a 2-3 fold rise in plasma renin activity, and a consequent rise in angiotensin II plasma concentration, in hypertensive patients.

1.10.2 Pharmacokinetics and Drug Metabolism

1.10.2.1 Absorption

Following oral administration, losartan is well absorbed, with systemic bioavailability of losartan approximately 33%. About 14% of an orally-administered dose of losartan is converted to the active metabolite, although about 1% of subjects did not convert losartan efficiently to the active metabolite.

1.10.2.2 Metabolism

Losartan is an orally active agent that undergoes substantial first-pass metabolism by cytochrome P450 enzymes. It is converted, in part, to an active carboxylic acid metabolite.

1.10.2.3 Excretion

The terminal half-life of losartan itself is about 2 hours, and that of the active metabolite, about 6-9 hours. The pharmacokinetics of losartan and this metabolite are linear with oral losartan doses up to 200 mg and do not change over time. Neither losartan nor its metabolite accumulates in plasma upon repeated once-daily administration, (Merck, 2012).

1.10.3 Mechanism of Action

Antagonizes angiotensin II by blocking the angiotensin type one (AT1) receptor.

1.10.4 Methods of analysis of losartan potassium

Literature reveals that the several analytical methods have been used for the qualitative studies of Losartan Potassium such as high performance liquid chromatography (HPLC), high performance thin layer chromatography (HPTLC) electrochemical radioimmunoassay , reverse phase high pressure liquid chromatography (RP- HPLC). Spectroscopic method was also conducted for determination of Losartan Potassium and UV spectroscopic method has been used for the simultaneous estimation of Losartan Potassium and Hydrochlorothiazide (HCT), (Azhar and Atya, 2011).

The official monograph BP 2014 used isocratic elution and mobile phase to determine losartan potassium, consisted of mobile phase; 3 volume of acetonitrile and 7 volume of a solution containing 0.39 % w/v of sodium dihydrogen orthophosphate and 0.35% v/v of triethylamine

adjusted to pH 7 with orthophosphoric acid, column (25cmx4.6mm) packed with octadecylsilyl silica gel for chromatography (5µm),1.5 ml/min flow rate and detection wavelength 250 nm at an ambient temperature, retention time about 7.0 min.

BP used a gradient RP-HPLC for Losartan potassium impurities and BP describe a potentiometric titration for assay of Losartan potassium as raw material. (BP, 2014).

USP described a HPLC method for assay and impurities of losartan potassium. (USP, 2014).

1.11 Aim and objectives

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose and meet current pharmaceutical regulatory guidelines i.e. (B.P, 2009), (USP, 2007), (ICH, 1995, 1997) and (EP, 2005).

A number of criteria such as specificity, linearity, precision, accuracy, sensitivity and robustness must be investigated in order to validate analytical methods.

The main objectives of this research are to develop safe, reliable and reproducible HPLC and UV spectroscopic methods, for determination of Atorvastatin calcium and Losartan potassium in bulk and pharmaceutical formulation.

To validate and develop a stability indicating HPLC method for each Atorvastatin calcium and Losartan potassium.

To report full stability profile for each of Atorvastatin calcium and losartan potassium.

To develop a simple analytical procedure for conducting routine and stability studies on atorvastatin calcium and losartan potassium even in presence of its decomposition products.

To study the photo thermal stability of atorvastatin and losartan potassium in solid form and aqueous state and investigate the effect of parameters as concentration, pH, temperature on the stability.

To study the effect of separately adding some excipients, that are of common use in pharmaceutical dosage form on the rate of atorvastatin calcium and losartan potassium thermal decomposition.

To identify major degradation products for Atorvastatin calcium and losartan potassium

To validate an UV spectrophotometric method using first derivative technique to analyze atorvastatin calcium and losartan potassium in formulation dosage form.

To reveal the efficiency of the new HPLC methods in terms of safety, economy, reliability and precision.

To study the kinetic reaction rate of degradation in order to determine half-life (t1/2).

To prove the linearity of all chromatography methods validated.

2. MATERIALS, INSTRUMENTS AND METHODS

2.1 Materials

2.1.1 Chemical solvents

Acetonitrile 99.9% HPLC grade from Sdfine- chem limited India.

Methanol HPLC grade 99.99% from Scharlau Spain, triethylamine from Sdfine-chem limited India, ethanol HPLC grade 99.0 from Merck (Darmstadt, Germany), hydrochloric acid 37% L.R lab(India),formic acid 99.0% from Sdfine chem limited India, Orthophosphoric acid 85 % LR from Sdfine-chem limited India and hydrogen peroxide solution 35% ph Nord Merck pharmaceutical India and Water HPLC grade Merck pharmaceutical India.

2.1.2 Chemical reagents

1-Atorvastatin calcium USP99.86%, batch number avkiala 87b was supplied by Zydus Cadila healthcare Limited India.

2-Losartan potassium EP/BP was supplied by HUNHN ZHEJINNG HUNHN Pharmaceutical CO.LTD. Batch Number C5455-13-236.China.

3- Citric Acid BP. JPT India.

4. Calcium carbonate BPJPT India.

5- Potassium dihydrogen orthophosphate, from Merck India.

6-Sodium dihydrogen orthophosphate from Merck India.

7-Sodium hydroxide pellets BP.BDH England.

8-Povidone K 30 USP JRS pharma India.

9- Mangessium stearate BP Calmags.BmbH India.

10-Microcrystalline cellulose BP.JSR pharma India.

11-Lactose BP Meggle India.

12-Maze starch BP.Roquette India

13-Talc powder. BP.IMERYS tall I India

14- Tween 80 BP.KOLB INDIA

15. Trisodium orthophosphate $12H_2O$. British drug hose England.

16 -Acdisol BP.JPT India

17-Carboxymethyl cellulose - (VIVA PUR 591) USP.JSR PHARMA India.

18- Mangessium hydroxide BP Taurus chemical (p) LTD India.

19-Potassium bromide (KBr) spectroscopic grade 99.5% from Mreck

Darmastadt German.

2.2 INSTRUMENTS

2.2.1 High Performance Liquid Chromatograph

The following HPLCs were used:

a) High performance liquid chromatograph, Sykam Germany HPLC consisting of the following items:

1-Isocratic pump type S1121, Solvent delivery system. Serial number 0117710.
2-UV-VIS Detector S3200 with absorbance range of 0.005-2.0(AUFS), serial number 010313.
3-Injector valve model S6020, serial number 01 30 12.
4-Windows 2010 software with signal channel serial port number N203644.
5. Autosampler serial number 016410.
6 -Column type ODS 250x4.6mm, 5µm.
b) High performance liquid chromatograph Jasco JAPAN consisting of the following items.

1. Interface box LC- Net-ADC serial number A026861868.

2. Line degasser DC-2080 serial numberF059160885.

3. Pump (RHPLC) Quaternary pump serial number A02476195.

4. HPLC autosampler serial number A007561675.

5. Column oven CO-4061 serial number A009561687.

6 Photo Diode Array detector MD-4010 serial number A00746166

7. Bottle stand BS-4000-1.

c) High performance liquid chromatograph SYKAM GEARMANY Chromostar HPLC consisting of the following items:

1. Quaternary pump S2100 solvent delivery system, serial number 014614.

2. Oven thermo controller, serial number010511.

3. Autosampler injector S5200, serial number014614.

4. Detector DAD S3210, serial number010215.

5 Chemostation software Chromostar 6.3.

2.2.2 Ultraviolet visible spectrophotometer

The following UV-VIS spectrometers were used:

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

Serial numberA11454601899CD

b).Jasco V630 spectrophotometer double beam serial number C239761148 Japan.

c) UV/VIS spectrophotometer: perkin Elmer type Lambda 12, USA.

Ultra violet radiation 254 nm, UV Lamp model 4492(England).

2.2.3 Infrared Spectrometer

Infrared spectrometer, TF/IR -4100 Fourier transform serial number B187361016 Jasco Japan.

2.2.4 General equipments

Stability chamber model number, NEC-2281210.India.

Water bath MPH, D3006, type 1083 Germany.

Analytical balance, CP1245 Sartorius max 120gm, drifting 0.1mg Germany.

Vacuum pressure Perkin Elmer.USA.

Ultrasonic micro clean- 109 India.

Stability chamber new tronic India.

2.3 Methods

2.3.1 Identification test for Atorvastatin calcium and losartan potassium

A standard IR runs a single spectrum. An FT-IR uses an interferometer and makes several scans and then uses Fourier Transforms to convert the interferogram into an infrared spectrum. IR used monochromatic light where FTIR used polychromatic. In IR only narrow wavelengths are identified but in FT-IR Wide wavelengths are identified using interferometer in place of grating. In IR single time scanning while in FT-IR number of scans more; scans 50 times in minute providing better resolution and compound using library database available.it is more sensitive.

The FTIR spectra of reference standard , test sample of Atorvastatin Calcium or losartan potassium were recorded with FT-IR spectrometer 4100 (Jasco ,Japan) by KBr disc method , prepared by finely grinding 1 part of a atorvastatin calcium or losartan potassium with about 250 part of dried potassium bromide. The mixture was compressed under 10 tons in vacuum pressure .Perkin Elmer.FT-IR computerized spectrometer was used to obtain IR spectra, along the wavelength range of 400 to 4000 cm^{-1},

with scanning speed of 2 mm/sec, at a resolution of 4 cm^{-1} using detector (TGS) triglycine sulfate.

2.3.2 The effect of solvent on UV spectrum of Atorvastatin calcium and losartan potassium.

Accurate weight of 0.109 gram (equivalent to 0.186 m mole) of Atorvastatin calcium and 0.54.3gram (equivalent to 0.24 m mole) of Losartan potassium was each transferred to dry 100 volumetric flask, and dissolved with (water, methanol, ethanol, acetonitrile, n.hexane, ethyl acetate Acetonitrile, 0.1M HCl, 0.1M NaOH, or chloroform solvent;1ml of the above solution was diluted with 50% methanol to obtain 10µg/ml of atorvastatin calcium or losartan potassium, and scanned over the range 200- 400nm.

2.3.3 Effect of pH on the UV spectrum of Atorvastatin calcium and Losartan potassium.

Accurate weight of 0.109 gram (equivalent to 0.186 m mole) of Atorvastatin calcium and 0.54.3gram (equivalent to 0.24 m mole) of Losartan potassium was each transferred to dry 100 volumetric flask, dissolved with (methanol for atorvastatin and water for losartan) and diluted to 100 volumetric mark with the respective solvent.1.0 ml of each solution had 10µg/ml .Each solution was scanned from 200 to400 nm then, one drop added of 0.1M NaOH or 0.1M HCl and scanned over 200 to 400nm.

2.3.4 Effect of pH value on the spectrum of Atorvastatin calcium and Losartan potassium.

Accurate weight of 0.109 gram (equivalent to 0.186 m mole) of Atorvastatin calcium and 0.54.3gram (equivalent to 0.24 m mole) of Losartan potassium was each transferred to 100 volumetric flask with methanol for atorvastatin and water for losartan. 1 ml was transferred in100 ml volumetric flask and the volume was completed to the mark with buffer pH range from 2 to 12, to obtain a concentration of 10µg/ml, the solution was scanned along the range 200 to 400nm.

Preparation of the approximate universal buffer solution was carried out according to the method described by: Carmody (1961) as shown intable (2.3.1).

Table (2.3.1) universal buffer solution

pH	Xml	pH	Xml	pH	Xml
2.0	195	5.5	126	9.0	69
2.5	184	6.0	118	9.5	60
3.0	176	6.5	109	10.0	54
3.5	166	7.0	99	10.5	49
4.0	155	7.5	92	11.00	44
4.5	144	8.0	85	11.50	33
5.0	134	8.5	78	12.00	17

I. The volume(X) contained 0.2M boric acid in 0.05M citric acid

II. The total volume was completed to 200ml volumetric flask with0.1M trisodium orthophosphate $12H_2O$.

III. Sodium hydroxide or orthophosphoric acid was used (if necessary) for adjustment to required pH –value.

2.3.5 Design and Validation of analytical methods

2.3.5.1 Method development and optimization

In this work HPLC method for determination of Atorvastatin calcium in tablets and their degradation products was developed and validated. The optimization of the method was done by selection of suitable solvents such as methanol and acetonitrile, different columns C8, C18, and detection wavelength and analyte concentration. The detection wavelength of 246 nm was selected after scanning the standard solution over the range 200-400 nm by using UV detector.

The traditional approach to HPLC optimization is to perform an experiment by trial and error or by change one control variable at time; such method can frequently require a very large number of experiments to identify the optimal condition. Recently computer assessed to HPLC separation has addressed the problem using factorial design strategies.

An eight- run ,2^3 factorial design of three factors at two level was set up to standardize the spectrographic condition which are likely to be employed .Percentage of acetonitrile in the organic phase (X1), proportion of orthophosphoric acid 0.1 %(X2) and flow rate (X3) as per 2^3 factorial design are represented in the table Factors and their corresponding levels as per 2^3 factorial design.

X1, X2 and X3 are independent factors whereas Y1, Y2 and Y3 are dependent factors.

Factors and their corresponding levels as per 2^3 factorial design Independent factors was tabulated in Table (2.3.5.1).

Table (2.3.5.1) The levels factors and their employed as per 2^3 factorial design

Factor	Level	
	-1	+1
Acetonitrile %	65	70
Buffer V/V	35	40
Flow rate ml/min	1.0	1.5

In this work a three factors with two level was applied to predict the retention behavior of Atorvastatin calcium and optimize their isocratic elution using acetonitrile as organic modifier and buffer as mobile phase table (2.35.1).

Table (2.3.5.2) Matrix for three factors (number of runs =8)

Experiment	Factor X1	Factor X2	Factor X3
Run1	-1	-1	-1
Run2	1	-1	-1
Run3	-1	1	-1
Run4	1	1	-1
Run5	-1	-1	1
Run6	1	-1	1
Run7	-1	1	1
Run8	1	1	1

-1 and 1 are symbols representing different levels in factorial design notation.

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice.

2.3.5.2 Development and Validation of an UV derivative spectrophotometric determination of Atorvastatin Calcium.

The derivative spectroscopy is a fast and intuitive methods for estimation of the number and the positions of the bands in various branches of molecular spectroscopy (UV-Vis-NIR, IR/Raman).

The derivative spectra crucially depends on the way of recording of the zero-order spectral curve. In the older instruments (prism monochromator) the measured spectra are function the energy (wavenumbers), while in all modern instruments (grating monochromator) the measured absorbance is linear to the wavelength scale. The change of the scale from wavenumbers to wavelengths leads to rising asymmetry of the energetically symmetric absorption bands (Antonov, 1997).

Derivative spectrophotometry is one of the advanced modern spectrophotometric techniques. It is based on so called derivative spectra which are generated from parent zero-order ones. The derivatisation of zero-order spectrum can lead to separation of overlapped signals, elimination of background caused by presence of other compounds in a sample. The mentioned properties can allow quantification of one or few analytes without initial separation or purification. Nowadays, this technique becomes very useful, additional tool which helps to resolve various analytical problems. It has found application in many fields of analysis (Talsky, 1994).

2.3.5.2.1 Selection of solvent

Atorvastatin was very soluble in methanol so after study the solubility profile of atorvastatin calcium in different solvents, methanol as a solvent was selected as common solvent for developing spectral characteristics.

2.3.5.2.2 Preparation of standard solution

Accurate weight of 43.46 mg (equivalent to 0.074 m mole) of Atorvastatin calcium was transferred into clean, dry100mL volumetric flask and dissolved in 50% of methanol, the above solution was diluted to obtain concentration of 10µg/ml of atorvastatin calcium, and this solution was scanned over the range of 200 to 400nm, using methanol 50% as blank, and then the wavelength selected.

2.3.5.2.3 Linearity and range

Serial dilution from the standard solution were taken in dry, clean 25ml volumetric, 2 ml,2.25,2.5,2.75 and 3.0 ml the volume was completed to the mark with 50% methanol to obtain a concentration of 16,18,20,22, and 24µg/ml , molar absorptivity can be determined.

2.3.5.2.4 Precision and Accuracy

Preparation of Standard sample solution:

Accurate weight of 43.46 mg (equivalent to 0.074 m mole) of Atorvastatin calcium standard (99.81% purity) was transferred into a 100 volumetric flask and the volume was completed with 50% methanol to obtain a concentration of10µg/ml.

2.3.5.2.5. Specificity and robustness

Assay of tablet formulation.

A commercially available of 43.46 mg (equivalent to 0.074 m mole) of Atorvastatin calcium tablet from different companies was dropped into 100ml volumetric flask and 50% methanol added. It was treated in ultrasonic bath for 15 min at 25 ^{0}C then was completed to the mark, filtrated through a whatmann filter to obtain a concentration of 10µg/ml for atorvastatin calcium.

2.3.5.2.6 Sensitivity

The sensitivity depends upon the experimental condition. The maximum sensitivity of which a method is capable is expressed in term of detection limit, sensitivity of atorvastatin was calculated in the range of a concentration 16-24µg/ml at 236 nm.

2.3.5.2.7 Force degradation study

Atorvastatin calcium was subjected to stress testing as per ICH recommended test condition. The drug subjected to acid hydrolysis by using 0.1M HCl, base hydrolysis 0.1M NaOH, oxidation 3% H_2O_2, thermal and photolysis.

2.3.5.3 Development and Validation of High performance Liquid Chromatographic Methods for determination of atorvastatin, method number 1

When new separation problem is faced, starting decisions concerning the type of stationary phase, solvent type and water phase nature should be made. According to lipophilic properties of the analyzed substances, acetonitrile was selected as organic modifier in the mobile phase, to fulfill the maximum efficiency of analytical method, critical optimization processes might be required, towards this, and the factorial design has been applied for optimizing analytical methods.

2.3.5.3.1 Determination assay of Atorvastatin calcium

Chromatographic separation was performed on SYKAM HPLC isocratic system equipped with a pump S1121 delivery system, variable-wavelength.

Chromatographic analysis was performed on Kromasil 100-5 C1825cmx4.6mm E82860 i.d., 5µm particle (column).Separation was achieved using mobile phase consist of 0.1% orthophosphoric acid: Acetonitrile (66:34) solution at flow rate 1.0ml/min. The eluent was monitored using UV detector at wavelength 246nm .The column was maintained at ambient temperature and injection volume of 20µl was used. The mobile phase was filtered through 045µm filter prior to use.

The chromatographic system used to perform development and validation of this assay method consisted of an Sykam Germany pump S2100 solvent delivery system , autosampler S5200 sample injector , S3210 UV/VIS detector and UV-visible double beam spectrophotometric (UV-1800 SHIMADZU Limited Japan.)

2.3.5.3.2. Preparation of mobile phase

Mobile phase consisted of 0.1% orthophosphoric acid: Acetonitrile (66:34) .The mobile phase was degassed by sonication before use.

2.3.5.3.3 Preparation of standard solution

Accurate weight 109 mg (equivalent to 0.186 m mole) of atorvastatin calcium transferred into 100ml volumetric flask, the solution was diluted with methanol to obtain stock solution of 1000µg/ml of atorvastatin calcium,

2.3.5.3.4 Specificity (ICHQ2B, 1997), (ICH, 2005).

A placebo blank containing critic acid 20mg, magnesium stearate 20mg, microcrystalline cellulose 20mg, lactose 20mg , tween 80 25mg, Acdisol 20mg, calcium carbonate 20mg , disodium phosphate 20mg and

magnesium hydroxide 20mg. The placebo blank solution was prepared to obtain a concentration of 10µg/ml.

2.3.5.3.5 Preparation of tablet solution

To determine the concentration of atorvastatin calcium in tablet dosage form (label 20 mg per tablet) 20 tablets were weighed, their mean weight was determined and they are finely powdered. An accurate weight of 43.46 mg (equivalent to 0.074 m mole) of atorvastatin transferred in 100ml volumetric flask and completed the volume with methanol the flask was sonicated for 15 min and the resulting solution was filtrated through 0.45µm membrane filter. Take apportion of solution to obtain a concentration 10µg/ml.

2.3.5.3 .6 Linearity and range

Different aliquots, 0.16, 0.18, 0.20, 0.22 and 0.24 ml of stock solution were taken in a series of 25 ml volumetric flasks and diluted up to the mark with diluent to get required concentration range of 80 %to 120%. The solution were then filtrated through 0.45µm nylon filter and injected into HPLC system.

Preparation of diluents: 90:10 of acetonitrile and water.

2.3.5.3.7 Accuracy

0.18, 0.2 and 0.22 ml of the stock solution was taken in triplicate in nine 25 ml volumetric flasks, and about 0.233g of placebo in it. Sufficient diluent was added to the flasks and sonicated for 20 min to dissolve. The volume was made up with diluent and mixed. The solution were filtrated through 0.45µm and injected into HPLC system. The chromatograms were recorded and the percentage recovery was calculated.

2.3.5.3.8 Precision

For the precision study, precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day) in triplicate. Repeatability refers to use of the analytical procedure over a short period of time that was evaluated by assaying the QC samples the same day. Intermediate precision was assessed by comparing the assays on different days (3 days) and % RSD calculated using 8µg/ml of test sample.

2.3.5.3.9 Stability of solution

A sample solution of 8µg/ml of drug were prepared by taken 0.18ml of standard solution in 25 ml volumetric flask and kept at room temperature. It was analyzed initially and at different time intervals day 1 and day 2.

2.3.5.3.10 Preparation of solution for degradation Studies:

2.3.5.3.10.1 Acid and base degradation

Accurate weight of 43.46 mg (equivalent to 0.074 mmole) of Atorvastatin calcium was dissolved with 10 ml in 100 volumetric flask, sonicateted for 15 minutes with intermittent shaking, add 5 ml of 0.1M of HCl or 5ml of 0.1M NaOH were added separately. The sample was heated on boiling water bath for 45 minutes, cool to room temperature and diluted to volume with diluent the sample was neutralized to pH 7 by adding 0.1M HCl or 0.1 M NaOH, mixed well. The acidic degradation and the alkaline forced degradation was performed in dark in order to exclude the possible degradation effect of light. This solution was filtered through 0.45um filter, 5ml of the filtrate was transferred to 25 ml volumetric flask, diluted to

volume with diluent (9:1) acetonitrile and water, mixed well and injected into the HPLC system.

2.3.5.3.10.2 Oxidation degradation

Accurate weight of 43.46 mg (equivalent to 0.074 m mole) of Atorvastatin calcium was dissolved with 10 ml of methanol into 100 volumetric flask, sonicateted for 15 minutes with intermittent shaking, 5 ml of 3% H_2O_2 was added. The sample was heated on boiling water bath for 45 minutes, cool to room temperature and diluted to volume with diluent, mixed well. This solution was filtered through 0.45um filter, 5ml of the filtrate was transferred to 25 ml volumetric flask, diluted to volume with diluent (9:1) acetonitrile and water, mixed well and injected into the HPLC system.

2.3.5.3.10.3 Thermal degradation

Accurate weight of 43.46 mg (equivalent to 0.074 m mole) of Atorvastatin calcium was dissolved with 10 ml of methanol into 100 volumetric flask, sonicateted for 15 minutes. The sample was heated on boiling water bath for 45 minutes, cool to room temperature and diluted to volume with diluent, mixed well. This solution was filtered through 0.45um filter, 5ml of the filtrate was transferred to 25 ml volumetric flask, diluted to volume with diluent (9:1) acetonitrile and water, mixed well and injected into the HPLC system.

2.3.5.3.10.4 Photolytic degradation

Accurate weight of 43.46 mg (equivalent to 0.074 m mole) of Atorvastatin calcium was dissolved with 10 ml into 100 volumetric flask, sonicateted for 15 minutes with intermittent shaking, and the sample was

kept under UV radiation at 254 nm for 16 hours, completed to the mark with diluent. This solution was filtered through 0.45um filter, 5ml of the filtrate was transferred to 25 ml volumetric flask, diluted to volume with diluent (9:1) acetonitrile and water, mixed well and injected into the HPLC system.

2.3.5.3.11 Robustness

According to ICH guideline robustness is a measure of the capacity of a method to remain unaffected by small, variation in parameters used in the method (Table 2.3.5.3.3).

Table (2.3.5.3.3) Method robustness with variable parameters

Flow rate(ml/min)			Wavelength			Mobile phase composition		
Original	used	Level	Original	used	Level	Original	used	Level
1.0	0.9	-0.1	246	244	-2.0	66:34	65:35	-1.0
	1.0	0.0		246	0.0		66:34	0.0
	1.1	+0.1		248	+2.0		67:33	+1.0

2.3.5.4 Apparatus and Chromatographic Conditions for validation method number 2

Samples were analyzed on the HPLC consist of SYKAM, pump S11211, solvent delivery system Autosampler S 5200, sample injector loop rheodyne 20µl and detector UV visible S3200.The UV detector was set at 246 nm , others apparatus included pH meter MATINI 180 Romania, oven Heraeus type VTR5022 Germany , Sartorius analytical balance CPA1245 and UV -1800 SHIMADZU spectrophotometer Japan.

The proposed RP-HPLC method utilized a 5µm particle Kromasil 100 column C18, (250mmx4.6mm) at ambient temprature . A 2^3 factorial design consisting of 3 factors at 2 levels was set up to standardize the

chromatographic condition. The mobile phase consisted of acetonitrile, formic acid 20 mM and 0.1% triethylamine(65:35v/v) ,the mobile phase was delivered isocratically at flow rate of 1.0 ml/min with UV detection at 246 nm, measurment were made with 20µl of injection loop.

2.3.5.4.1 Preparation of Standard Solution

All parameters validation are carried out according to the method described in validation method No 1(2.3.5.3): as follow (2.3.5.3.1), (2.3.5.3.2), (2.3.5.3.3), (2.5.5.3.4), (2.3.5.3.5), (2.3.5.3.6), 2.3.5.3.7), (2.3.5.3.8), (2.3.5.3.9) and (2.3.5.3.10).

2.3.5.4 .2 Robustness

Determination of robustness is a systematic process of varying a parameters and measuring the effect on the method by monitoring system suitability. The robustness of the method was illustrated in the table (2.3.5.4.1)

Table (2.3.5.4.1) Method robustness Test

Flow rate(ml/min)			Mobile phase composition		
Original	used	Level	Original	used	Level
1.0	0.9	-0.1	65:35	64:35	-1.0
	1.0	0.0		65:35	0.0
	1.1	+0.1		66:35	+1.0

2.3.5.5. Development and validation of an UV derivative spectro-photometric for determination losartan potassium

2.3.5.5.1 Solubility determination

Solubility of Losartan potassium was determined in different solvents. Losartan was found to be soluble in water and methanol.

2.3.5.5.2 Stock solution and standard

Accurate Weight of 54.46 mg (equivalent to 0.118 m mole) of losartan potassium was transferred into clean, dry100 mL volumetric flask and dissolved in 50% of methanol, the above solution was diluted to obtain concentration of 10µg/ml of atorvastatin calcium, and this solution was scanned along the range of 200 to 400nm, using methanol 50% as blank, and then the wavelength selected.

2.3.5.5.3 Linearity and range

Standard stock solution of losartan potassium was diluted to yield different concentrations of 4-14 µg/ml .The absorbance was measured at 234nm, the standard curve was plotted against concentration versus absorbance of dilution, and the concentration obeyed Beer's law.

2.3.5.5.4 Accuracy and precision

Accuracy was assessed using over three concentration levels covering the specific range 4-14 µg/ml; accuracy was reported as percent recovery by the assay

2.3.5.5.5 Molar absorptivity and Sandall's sensitivity

Serial dilutions from the stock solution were taken in dry, clean 25ml volumetric flask to obtain a concentration of 4,6,8,10,12 and 14 µg/ml.

2.3.5.5.6. Specificity

The specificity test of the method demonstrated that the excipients from marketed tablets did not interfere with the active ingredient.

Accurate weight of 54.4 mg of losartan potassium tablets equivalent to 50mg was transferred into dry clean 100ml volumetric flask, dissolved in 50% methanol and filtered through whatmann filter paper. 1.0 ml was pipette out to obtain a concentration of 10µg/ml of sample and measured at 234 nm.

2.3.5.5.7 Recovery study

To study the accuracy and repeatability of the proposed method, recovery experiments were carried out by adding a known amount of drug to preanalysed sample and the percentage recovery was calculate.by using 10µg/ml.

2.3.5.5.8 Precision

The precision of the assay were performed by repeatability (intraday) and intermediate precision in triplicate sample; 10µg/ml used, RSD% was reported.

2.3.5.6. Development and Validation HPLC method for analysis of Losartan potassium method No 1

The HPLC analysis was performed on reversed phase high performance liquid chromatograph system with isocratic elution mode using a mobile phase of acetonitrile: buffer of 10.5mM of disodium hydrogen phosphate and 10mM potassium dihydrogen phosphate (40:60)

on Kromasil 250x4.6mm100-5 C1825cmx4.6mm E82860 i.d, 5µm particle, with 1.0ml/min flow rate at 250nm using UV detector.

2.3.5.6.1 Preparation of mobile phase and stock solution

Mobile phase: The mobile phase containing 40:60 Acetonitrile: Buffer consist of 10.5mM of disodium hydrogen phosphate and 10mM potassium dihydrogen phosphate, pH 3.5 adjusted by 10 % Orthophosphoric acid. The mobile phase sonicated for 10min and filtrated through 0.45µm membrane filter which was used as a mobile phase.

Accurate weight of 109 mg (equivalent to 0.24 m mole) of losartan potassium into 100ml volumetric flask, the solution was diluted with methanol to obtain a concentration of 1000µg/ml of losartan potassium.

2.3.5.6.2 Linearity and range

Different aliquots, 0.16, 0.18, 0.20, 0.22 and 0.24 ml of stock solution were taken in a series of 25 ml volumetric flasks and diluted up to the mark with diluent to obtain required a concentration range of 80 % to 120%. The solution were then filtrated through 0.45µm nylon filter and injected into HPLC system.

2.3.5.6.3 Accuracy and Recovery

The recovery was performed at three levels 80%, 100% and 120% of the label claim of losartan potassium

Accurate weight of 54.4 mg (equivalent to 0.12 m mole) of losartan potassium into 100ml volumetric to obtain 500µg/ml, the amount of losartan potassium at three levels were added and the percentage recoveries were calculated from the calibration curve.

2.3.5.6.4 Precision (repeatability)

Repeatability precision is expressed as standard deviation of the analytical results of assay of atorvastatin concentration 100% in six replicate injections

2.3.5.6.5 Robustness

Robustness accepted when the system suitability parameters passed for all the conditions; all known and unknown impurities should be separated from each other in sample spiked with impurities. The robustness of the method was illustrated in the table (2.3.5.6.1).

Table (2.3.5.6.1) Method robustness test

Flow rate(ml/min)			pH of mobile phase		
Original	used	Level	Original	used	Level
1.0	0.9	-0.1	3.5	3.4	-0.10
	1.0	0.0		3.5	0.0
	1.1	+0.1		3.6	+0.1.0

2.3.5.6.6 System suitability

Once a method or system has been validated the task becomes one of routinely checking the suitability of the system to be performed within the validation limits;for this a concentration of 100% was used.

2.3.5.6.7 Preparation of degradation samples

2.3.5.6.7.1 The effect of acid or base on stability of losartan potassium

Accurate weight of109mg (equivalent to 0.118 m mole) of losartan potassium was dissolved with 10 ml in 100 volumetric flask, sonicateted for 15 minutes with intermittent shaking; 5 ml of 0.1M of HCl or 5ml of

0.1M NaOH were added separately. The sample was heated on boiling water bath for 45 minutes, cooled to room temperature and diluted to volume with diluent .The sample was neutralized to pH 7 by adding 0.1M HCl or 0.1 M NaOH and mixed well. The acidic degradation and the alkaline forced degradation were performed in the dark in order to exclude the possible degradation effect of light. This solution was filtered through 0.45um filter, 5ml of the filtrate was transferred to 25 ml volumetric flask, diluted to volume with diluent (9:1) acetonitrile and water and mixed well and injected into the HPLC system.

2.3.5.6.7.2 Oxidation degradation

Accurate weight of109 mg (equivalent to 0.118 m mole) of losartan potassium was dissolved with 10 ml of methanol into 100 volumetric flask, sonicateted for 15 minutes with intermittent shaking; 5 ml of 3% H_2O_2 was added. The sample was heated on boiling water bath for 45 minutes, cooled to room temperature and diluted to volume with diluent and mixed well. This solution was filtered through 0.45um filter. 5ml of the filtrate was transferred to 25 ml volumetric flask, diluted to volume with diluent (9:1) acetonitrile and water, mixed well and injected into the HPLC system.

2.3.5.6.7.3 Thermal degradation

Accurate weight of109mg (equivalent to 0.118 m mole) of losartan potassium was dissolved with 10 ml of methanol into 100 volumetric flask, sonicateted for 15 minutes. The sample was heated on boiling water bath for 45 minutes, cooled to room temperature and diluted to volume with diluent and mixed well. This solution was filtered through 0.45um filter.5ml of the filtrate was transferred to 25 ml volumetric flask, diluted

to volume with diluent (9:1) acetonitrile and water and mixed well and injected into the HPLC system.

2.3.5.6.7.4 Photolytic degradation

Accurate weight of109 mg (equivalent to 0.118 m mole) of losartan potassium was dissolved with 10 ml into 100 volumetric flask, sonicateted for 15 minutes with intermittent shaking. The sample was kept under UV radiation at 254 nm for 16 hours and completed to the mark with diluent. This solution was filtered through 0.45um filter, 5ml of the filtrate was transferred to 25 ml volumetric flask, diluted to volume with diluent (9:1) acetonitrile and water, mixed well and injected into the HPLC system.

2.3.5.7 Method Validation No 2

The mobile phase consisted of acetonitrile, 0.05% orthophosphoric acid plus 0.05% triethylamine at ratio of 40:60, the flow rate was 1.0ml/min, wavelength was 225 nm, injection volume was 20µl loop and separation was achieved on column C_{18} in ambient.

All components of method validation are carried out as described in (2.3.5.6.1), (2.3.5.6.2), (2.3.5.6.3), (2.3.5.6.4), (2.3.5.6.5), (2.3.5.6.6) and (2.3.5.6.7).

2.3.5.7.1 Robustness

Result from the testing of robustness of method by changing the flow rate, mobile phase composition and wavelength (the concentration of the solution analyzed was 10µg/ml of Losartan potassium) as .tabulated in table (2.3.5.7.1.).

Table (2.3.5.7.1) Robustness of method validation

Flow rate(ml/min)			mobile phase composition			Wavelength		
Original	used	Level	Original	used	Level	Original	used	Level
1.0	0.95	-0.05	40:60	39:61	-1.0	225	220	-5.0
	1.0	0.0		40:60	0.0		225	0.0
	1.05	+0.05		41:59	+1.0		230	+5.0

2.4 The effect of buffer on solubility of atorvastatin calcium or losartan potassium

Accurate weight of 109.0 mg (equivalent to 0.186 m mole) of atorvastatin calcium or 54.63 mg (equivalent to 0.12 m mole) of losartan potassium was dissolved with 10 ml of methanol in 100 volumetric flask and sonicateted for 15 minutes, the volume was completed to the mark with methanol 10ml of each pipette into 100ml volumetric flask. The volume was completed to the mark with buffer pH 2 to 12 for atorvastatin and losartan .The solution was scanned over the rang 200 to 400nm.

2.4.1The effect of pH on losartan potassium as function of pH.

Accurate weight 54.63 mg (equivalent to 0.12 mmole) of losartan potassium was dissolved with 10 ml of methanol in 100 volumetric flask and sonicateted for 15 minutes, the volume was completed to the mark with methanol 10ml of each pipette into 100ml volumetric flask. The volume was completed to the mark with buffer pH 4, 5, 6, 6.8 and pH 7.The solution was scanned over the range 200 to 400nm.

2.5 The effect of excipient in reaction rate of thermal decomposition of atorvastatin calcium or losartan potassium at of 80 ^{0}C

The required quantities of excipients was weighed and transferred into 100ml volumetric flask containing 50: 50 methanol: water plus 109.0

mg (equivalent to 0.186 m mole) of atorvastatin calcium or 54.63 mg (equivalent to 0.12 m mole) of losartan potassium and heated in water-bath thermostated at 80 ^{0}C.Aliquots were withdrawn at 0, 10, 20,30,40,50, and 60 minutes into small beaker and analyzed by proposed method.

2.6 Accelerating stability studies of atorvastatin calcium or losartan potassium

Five grams of each sample of atorvastatin calcium or losartan potassium were kept at stability chamber as per ICH guideline of 40 ^{0}C and 75±5 % RH up to 6 months; 43.36 mg(equivalent to then 0.074 m mole) of atorvastatin calcium and 54.46 mg (equivalent to 0.118 m mole) of each atorvastatin calcium or losartan potassium of standard test and test sample was accurately weighed and transferred into separated 100 volumetric flask and added 10ml of methanol sonicated for 15 minutes and diluted to the mark to obtain a concentration of 10µg/ml with diluent and analyzed by proposed HPLC methods.

2.7 Thermal degradation of atorvastatin calcium or losartan potassium

Accurate weight of 43.36 mg (equivalent to 0.074 m mole) of atorvastatin calcium or 54.46mg (equivalent to 0.118 m mole) of losartan potassium was transferred into 100ml volumetric flask; 10ml of methanol was added and sonicated for 15 minutes with intermittent shaking. The sample was heated on boiling water bath for 45 minutes, cooled to room temperature and diluted to the volume with diluent and mixed well. The solution was filtrated through 0.45µm filter; 5ml of the filtrate was transferred to 25 ml volumetric flask, diluted to volume with diluent (9:1) acetonitrile and water, mixed and injected into the HPLC system.

2.8 The effect of natural daylight on instability of Atorvastatin calcium or Losartan potassium

Accurate weight of 43.36 mg (equivalent to 0.074 m mole) of atorvastatin calcium or 54.46mg (equivalent to 0.118 m mole) of losartan potassium was transferred to 100ml volumetric flask and completed to the mark with methanol for atorvastatin and distilled water for losartan potassium. The solution was directly exposed to sunlight, aliquots were withdrawn at each one hour, 2, 3,4,5,6 7and 8 hours. The solution was filtrated through 0.45µm filter, 5ml of the filtrate was transferred to 25 ml volumetric flask, diluted to volume with diluent (9:1) acetonitrile and water, mixed and injected into the HPLC system.

2.9 Thermal decomposition of an atorvastatin calcium or Losartan potassium in acid and base medium at 80 ^{0}C controlled temperature

Accurate weight of 43.36 mg (equivalent to 0.074 m mole) of atorvastatin calcium or 54.46mg (equivalent to 0.118 m mole) of losartan potassium was transferred to 100ml volumetric flask and completed to volume mark with methanol for atorvastatin and distilled water for losartan potassium.30 ml of the solution of both atorvastatin calcium and losartan potassium was added to 70 ml of 0.1 M HCl or 0.1M NaOH ,respectively, heated in thermosted water bath at temperature of 80 ^{0}C .Aliquots were withdrawn at 0,10,20,30,40,50 and 60 minutes for atorvastatin in base media and losartan potassium in acid media and 0,10,20 30 minutes for atorvastatin in acid media and losartan in base media , cooled at room temperature and diluted to obtain a concentration of 10µg/ml of each drug and then analyzed by HPLC proposed methods.

2.10 The effect of controlled temperature of 100 ^{0}C on stability of atorvastatin calcium

Five grams of atorvastatin calcium was placed in thermostatic oven; portions of the sample were withdrawn at different days, zero day, day 3 and day 4.Sample was treated to obtain a concentration of10µg/ml and then analyzed by HPLC method No1.

2.11 The effect of UV radiation on instability of losartan potassium

Accurate weight of 54.46mg (equivalent to 0.118 m mole) of losartan potassium was transferred to 100 ml volumetric flask, dissolved with water, and the solution was directly exposed to the UV radiation at 254nm .The solution withdrawal at different days, day 1, 2, 3 and day 6 respectively and analyzes by method No 2.

2.12The effect of thermal decomposition of Losartan potassium in an acid and base media

Accurate weight of54.46mg (equivalent to 0.118 m mole) of losartan potassium and transferred into dry clean 100ml volumetric flask, 30ml of the solution added to 70ml of 0.1 M NaOH or 0.1M HCl and refluxed for 2 hours, cooled , neutralized to pH 7 and analyze by proposed HPLC method No 2.

2.13 Comparison between the official BP method and validation methods No. 1 and No.2 for determination losartan

Table (2.13.1) shows the comparison of the parameters with developed validation method for the determination of losartan potassium with those of the official BP method.

Table (2.13.1) Comparison between BP 2014 method and validation methods No .1 and No. 2

Parameters	Official method	Method No 1	Method No 2
Mobile phase	3volume of acetonitrile ,7 volume of solution containing 0.35% v/v of triethylamine , 0.39% w/v sodium dihydrogen orthophosphate and adjusted to pH 7 with orthophosphoric acid	10mM of disodium hydrogen phosphate 10mM potassium dihydrogen Phosphate and pH3.5 adjusted by ortho-phosphoric Acid Acetonitrile : Buffer (40:60 v/v)	Acetonitrile,0.05% orthophosphoric acid+0.05% triethylamine (40:60 v/v)
Flow rate	1.5 ml/min	1.0 ml/min	1.0 ml/min
UV detection	250 nm	250nm	225nm
Injection volume	10µl	20µl	20µl
Column	C_{18} (250 cm x4.6 , 5 µm)	C_{18} (250x4.6 mm,5µmm)	C_{18} (250x4.6 mm,5µmm)
Temperature	Ambient	Ambient	Ambient

RESULTS AND DISSCISSION

3.1 Identification

3. 1.1 Identification test for atorvastatin calcium

The IR spectrum of atorvastatin calcium showed characteristic peak at 2970 cm^{-1} (C- H) a aliphatic stretching, 1317.14 cm^{-1} (C-N) stretching, 3055.66 cm^{-1} (O-H) alcohol group , 3397.96 cm^{-1} (N-H) stretching, 1594.84 cm^{-1} due to band of (C=C) bending of aromatic , 696.95 cm^{-1} stretching of (C-F) and the very important region between 1650 -1800 cm^{-1} showed the presence of (C=O) carbonyl group due to amide in atorvastatin structure. The IR represented frequencies of the modes are consistent of losartan calcium reference standard and test sample. With vibrational data in Table (3.1.1.).

From the I.R spectrum of atorvastatin calcium Figure (3.1.1), (3.1.2) and Table (3.1.1), the peak assignment agree to that of functional group conform the structure of atorvastatin calcium reference standard.

Table (3.1.1.) IR frequencies of Atorvastatin Calcium

Band frequencies of standard cm^{-1}	Band frequencies of test sample cm^{-1}	Band intensity	Causative functional group	Vibration mode
3406.64	3397.96	strong	N-H	Stretching
3055.66	3055.66	strong	O-H	Stretching
2969.84	2970.80	strong	C-H	Stretching
1650.77	1650.77	Strong	C=O	Stretching
1594.84,1524.45	1594.84,1524.45	medium	-C=C	Bending
1435.74	1435.74	medium	Aliphatic C-H	Bending
1158.04	1159.01	strong	C-O	Stretching
842.74,746.31	842.74,746.32	strong	Aromatic C-H	Bending
691.35	691.34	medium	C-F	Stretching
1315.21	1317.14	medium	C-N	Stretching

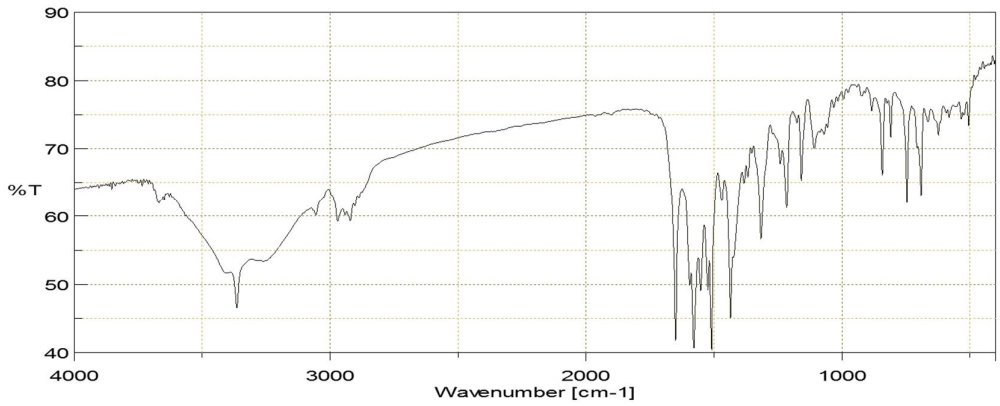


Figure (3.1.1.) I.R spectrum of Atorvastatin calcium reference

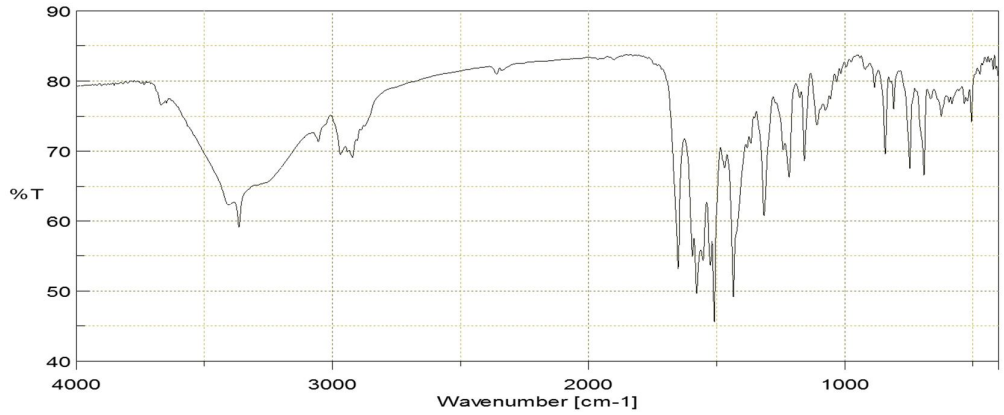


Figure (3.1.2) I.R spectrum of Atorvastatin calcium test sample

3.1.2. Effect of solvents on the UV spectrum of atorvastatin calcium

Majority of UV-visible spectrophotometric measurements require the analyte(s) to be dissolved in an appropriate solvent. Water and many organic solvents such as methanol, ethanol, acetonitrile, cyclohexane, dimethylformamide (DMF), dimethylsulfoxide (DMSO), etc. are used for this purpose. Solvents should dissolve the sample well, be optically transparent (not absorb incident radiation), and chemically inert and pure (Chandrasekhr *et al*., 2006).

Methanol selected as suitable solvent for developing spectral characteristics of the drug .The selection was made after assessing the solubility of drug in different solvents Figure (3.3.1).

Atorvastatin calcium in different solvents its absorbance in the following order Methanol> Ethanol >Acetonitrile, the chromophores in all solvents intact, table (3.2.2), Figure (3.2.2) and Figure (3.3.1).

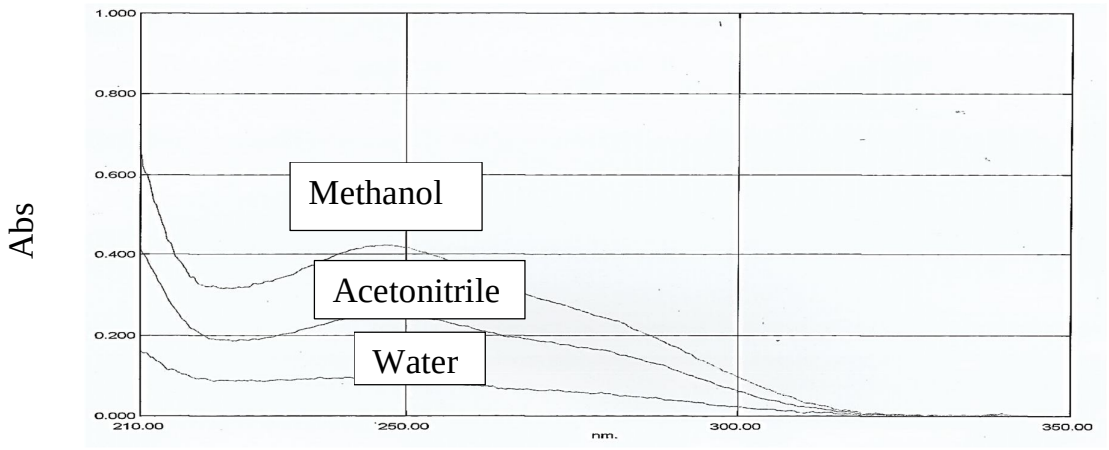


Figure (3.1.3) Solvents effect on UV spectrum of atorvastatin

Table (3.1.2) Solvents effect on UV spectrum of Atorvastatin calcium

no	Solvent	Absorbance246nm	Absorbance225nm
1	Water	0.082	0.041
2	Methanol	0.438	0.238
3	Ethanol	0.434	0.332
4	Acetonitrile	0.288	0.265
5	n. hexane	0.014	0.001
6	Ethyl acetate	0.266	0.225
7	Chloroform	0.398	0.300

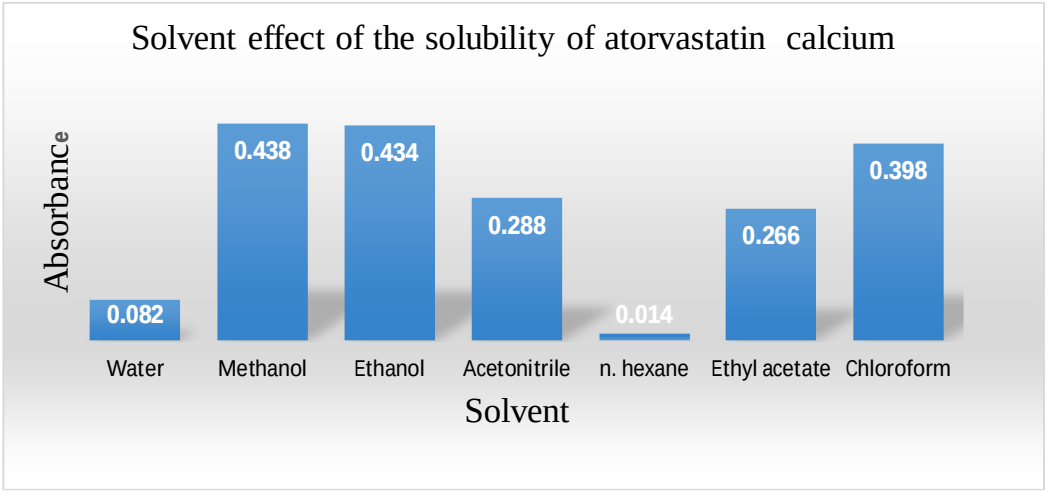


Figure (3.1.4) Solvent effect of absorption of atorvastatin calcium

3.1.3. Effect of pH on UV spectrum of atorvastatin calcium

Atorvastatin calcium has maximum absorbance in acid media at wavelength 246 nm Figure (3.1.5), and Table (3.1.3), the chromophores intact in extreme acid media or base.

Table (3.1.3.) Absorbance of atorvastatin in methanol, 0.1M HCL and 0.1M NaOH

Solvent	Absorbance 246nm	Absorbance 203nm
Methanol	0.841	1.735
0.1M HCl	0.858	1.777
0.1M NaOH	0.815	1.742

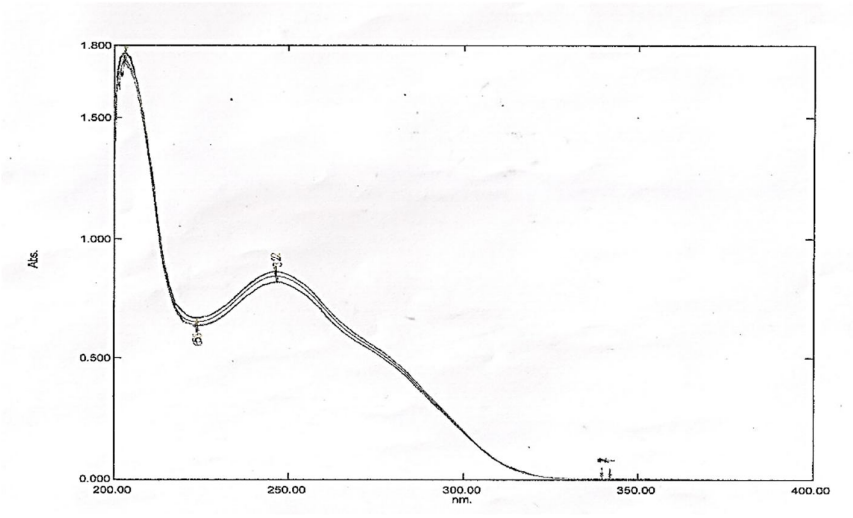


Figure (3.1.5) Effect of pH on atorvastatin spectrum

3.1.4 I.R identification test for losartan potassium

Losartan potassium is 2-butyl-4-chloro-L-[[20- (lH-tetrazol-5-yl)[l,l0-biphenyl]-4-yl]methyl]-lH-imidazole-5-methanol, gives one broad peak at 3200.29 cm-1 and twin peaks at 996 and 1,010 cm-1 owing to

tetrazole and gives one peak at 1459.85 cm-1 because of imidazole, free O-H broad peak observed at 3200.29cm^{-1} , C-O stretching was observed at 1074.16,N-H stretching was evident at 2956.34 cm^{-1}, C=N stretching at 1422.24 cm^{-1}, C-N stretching of tertiary amine was observed at 1259.29 cm^{-1} and aryl chloride was observed at 1112.73 cm^{-1}. The IR spectrum of the pure Losartan Potassium reference standard recorded by FTIR spectrometer is shown in Figure (3.1.6) and test sample figure (3.1.7),reference standard and sample test was comply functional group frequencies of Losartan Potassium are shown in Table (3.1.4.).

Table (3.1.4) Frequencies of losartan potassium

Band frequencies of standard cm^{-1}	Band frequencies of test sample cm^{-1}	Band intensity	Causative functional group	Vibration mode
3196.42	3200.29	Broad	O-H	Stretching
2956.34	2956.34	Strong	N-H	Stretching
1580.38,1551.4	1580.38,1552.42	strong	C-C	Stretching
1459.85	1459.85	medium	C-H/CH3	Bending
1423.20	1422.24	medium	C=N	Stretching
1074.16	1074.16	strong	C-O	Stretching
2956.54,2924.52	2956.34,2929.34	medium	Ar-C-H	Stretching
1260.24	12592.9	medium	C-N	Stretching
1113.33	1112.73	medium	Ar-Cl	Stretching

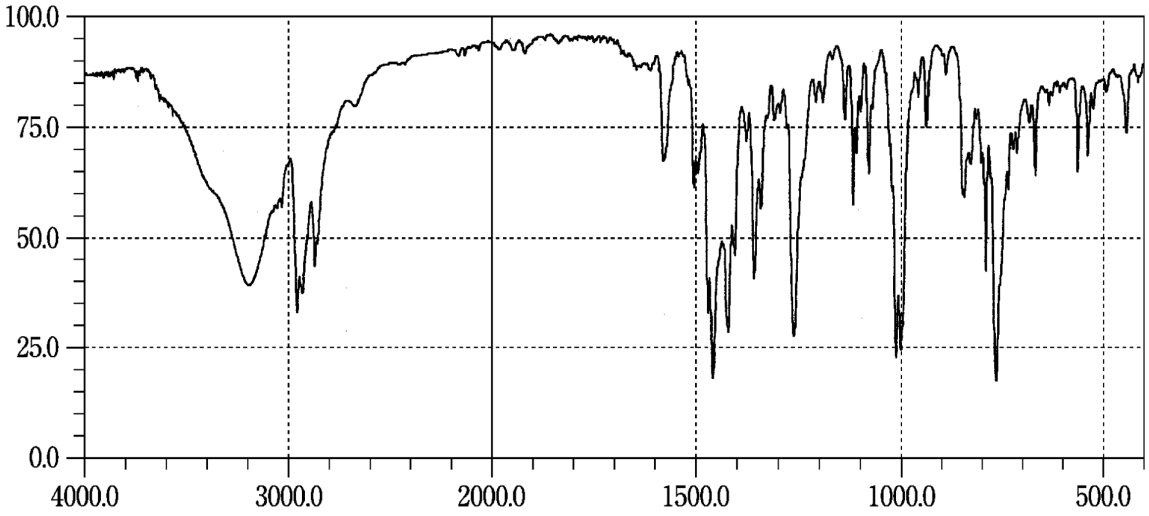


Figure (3.1.6) IR spectrum of Losartan potassium reference standard

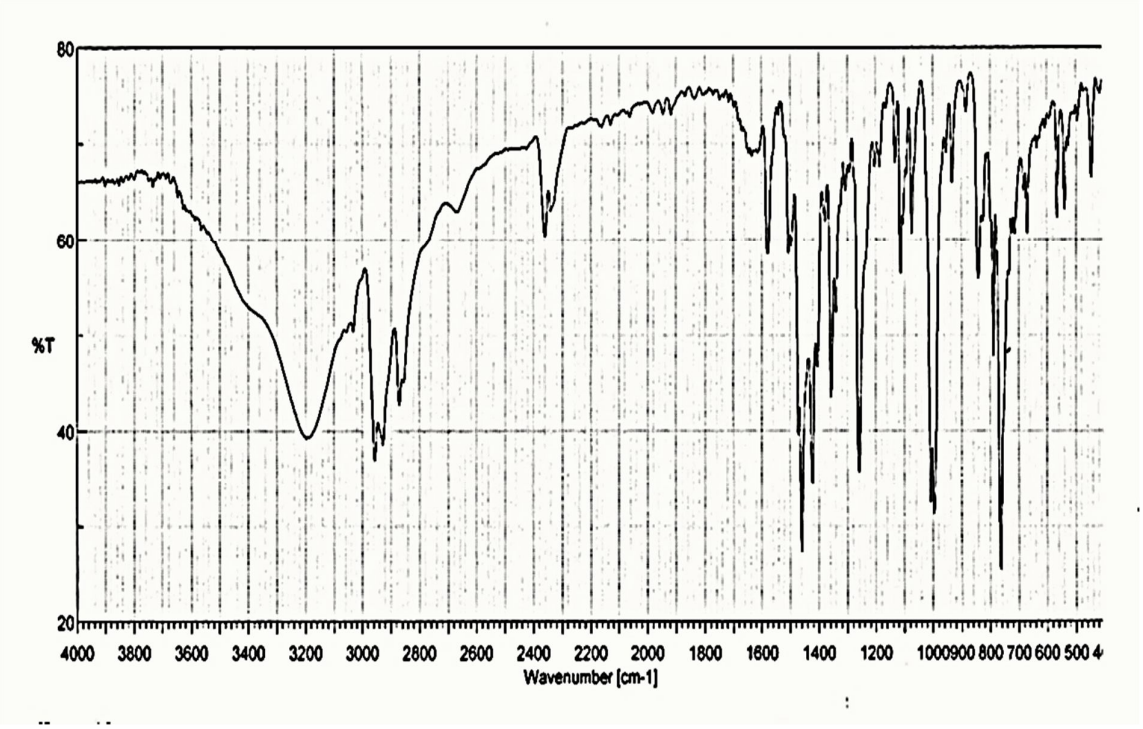


Figure (3.1.7) IR spectrum of Losartan potassium test sample

3.1.5 Effect of solvents on UV spectrum of losartan potassium calcium

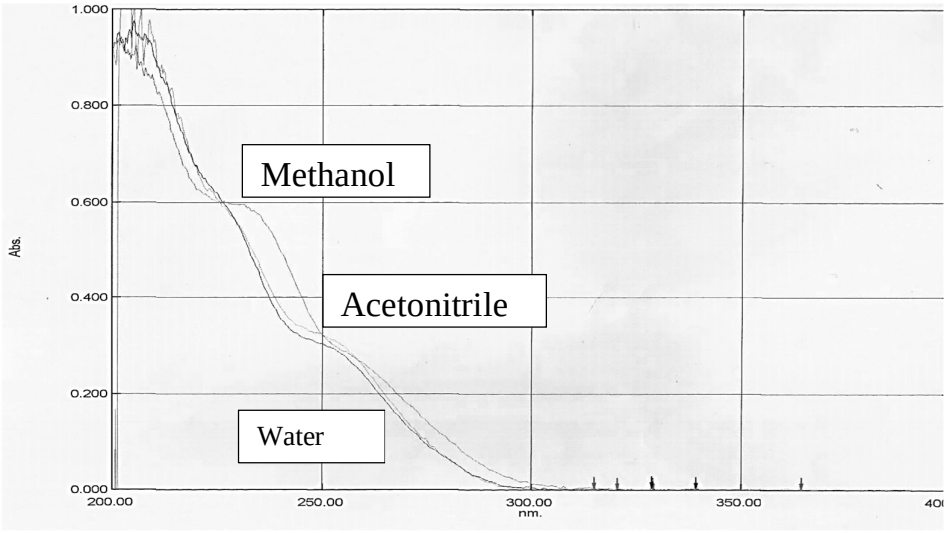


Figure (3.1.8) UV spectrum of Losartan potassium in different solvents

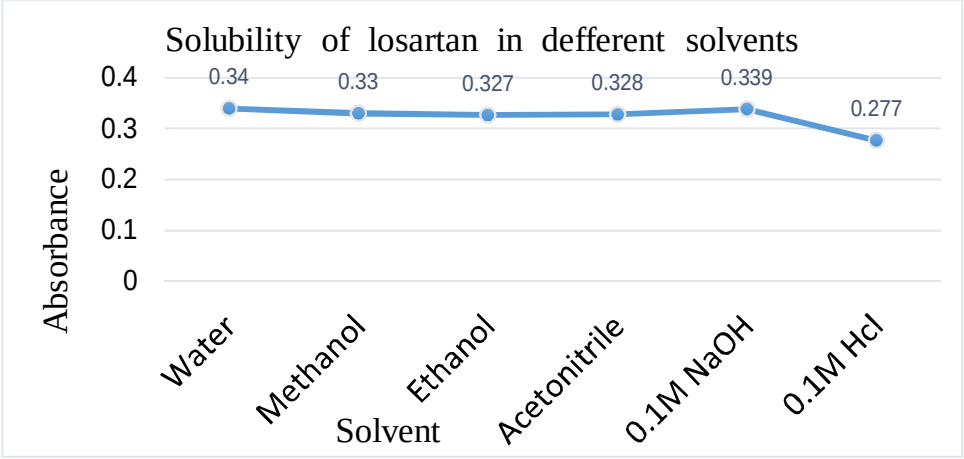


Figure (3.1 .9) the effect of solvent on losartan potassium absorbance

Table (3.1.5) solubility of losartan and pH influence

Buffer	λ1	ABS	λ 2	ABS	λ 3	ABS
pH 4	205nm	1.026	225nm	0.683	254	0.286
pH5	205nm	1.033	225nm	0.711	254	0.337
PH6	205nm	1.095	225nm	0.701	254	0.338
PH 6.8	205nm	1.12	225nm	0.720	254	0.341
pH 7	205nm	1.069	225nm	0.688	254	0.344

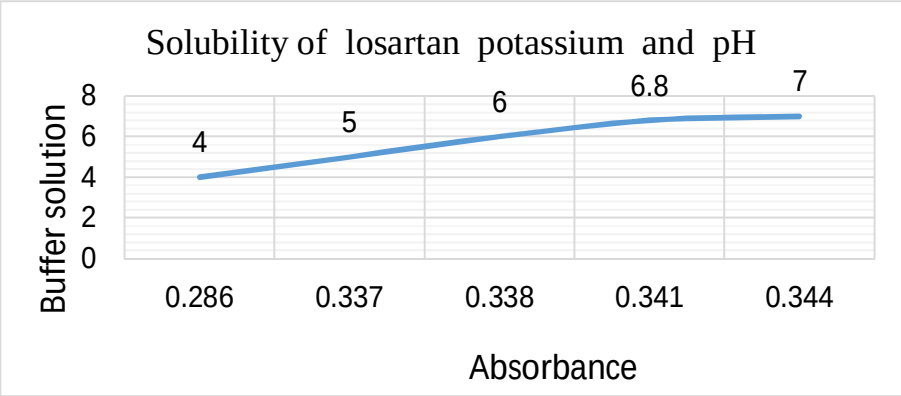


Figure (3.1.10) the solubility of losartan potassium as function of pH

3.1.6 The effect of pH on UV spectrum of losartan potassium

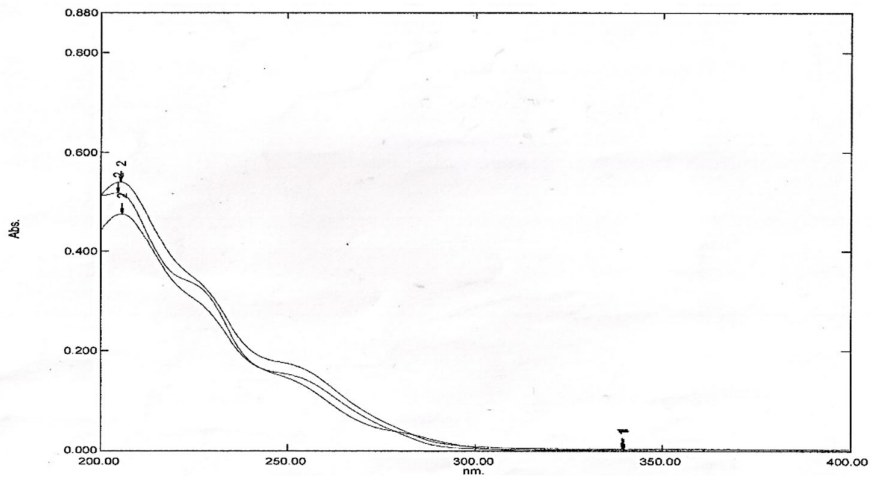


Figure (3.1.11) Effect of pH on UV spectrum of losartan potassium.

Table (3.1.6) Effect of PH on UV Spectrum of losartan potassium

Solvent	Absorbance at 205
Water	0.541
0.1M HCl	0.518
0.1M NaOH	0.476

3.1.7 Effect of solvents on solubility of Losartan potassium

Many organic solvents were used for dissolving losartan potassium, the solvents can be arranged in order of their absorbance as follow: water, methanol, acetonitrile, sodium hydroxide, and ethanol and 0.1M hydrochloric acid, so water was chosen as the best solvent, Figure (3.1.11).

Many organic solvents were using for dissolving losartan potassium, the solvents can be arranged in order of their absorbance as follow : water, methanol, acetonitrile, sodium hydroxide , ethanol and 0.1M hydrochloric acid, so water can was chosen as the best solvent, table (3.1.6).

Selection of solvents was made after assessing the solubility of losartan potassium in different solvents. The ultraviolet absorption spectrum of losartan in water, methanol, ethanol, and acetonitrile, 0.1M NaOH and 0.1M HCl is shown in figure (3.1.8) and table (3.1.9), in deferent solvents the chromophoric system was not changed, the absorbance was recorded as water, methanol, and acetonitrile.

Losartan potassium exhibits maximum absorbance at 205 nm due to tetrazole ring (π-π^*) in UVwhich causes bathochromic shift or blue shift, (Lachman, 1986).

The solubility of a substance fundamentally depends on the solvent used as well as on temperature and pressure. The extent of solubility of a substance in a specific solvent is measured as the saturation concentration

where adding more solute does not increase its concentration in the solution. The solubility of a weakly acidic and weakly basic drug can be increased if the drug remains in ionized state (Owen, 2000).

Most of the drugs are weak acids or weak bases; drugs with low water solubility will be difficult to incorporate into sustained release mechanism

For a drug with high (Losartan potassium) solubility and rapid dissolution rate, it is often quite difficult to retard its dissolution rate. A drug of high water solubility can dissolve in water or gastrointestinal fluid readily and tends to release its dosage form in a burst and thus is absorbed quickly leading to a sharp increase in the blood drug concentration compared to less soluble drug. It is often difficult to incorporate a highly water soluble drug in the dosage form and retard the drug release especially when the dose is high (Brahmankar, 2009).

In simple terms the solubility of acidic compounds increases as the pH of the solution is increased (above the pKa) and the solubility of basic compounds increases as the pH is lowered below the pKa. (Kamerzell *et al.*, 2011).

It has been observed that losartan potassium dissolved well in water, methanol, ethanol and it's practically soluble in organic solvents, the solubility increased when the pH increased Figure (3.1.10), so that Losartan potassium was pH dependent.

3.2 Methods Validation

3.2.1 Development and Validation of an UV derivative spectr-ophotometric determination of Atorvastatin Calcium

Derivative spectrophotometry can be very useful additional tool which helps to solve some complicated analytical problems. Mathematical processing of spectra is very easy to use as modern spectrophotometers are computer controlled and their software are equipped in derivatisation unit. A proper selection of mathematical parameters gives profits in improved selectivity, sometimes sensitivity and in simplification of analytical procedure. Derivative spectrophotometry (DS) has been introduced for the resolution of overlapping peaks. DS method has been widely used to enhance the signal and resolve the overlapped peak-signals due to its advantages in differentiating closely adjacent peaks, and identifying weak peaks obscured by sharp peaks.

The main disadvantage of UV derivative is its dependence on instrumental parameters like speed of scan and the slit width. The instrumental conditions of recording parent zero-order spectrum have strong influence on the shape and intensity of its derivative generations (Joanna, 2012)..

3.2.1.1 Selection of solvent

Atorvastatin was very soluble in methanol so after study the solubility profile of atorvastatin calcium in different solvents, methanol as solvent was selected as common solvent for developing spectral characteristics

3.2.1.2`Selection of wavelength

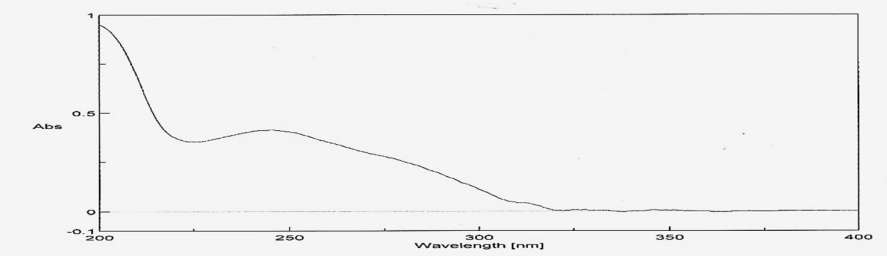


Figure (3.2.1.1) Zero order UV Spectrum of Atorvastatin calcium in 50% methanol

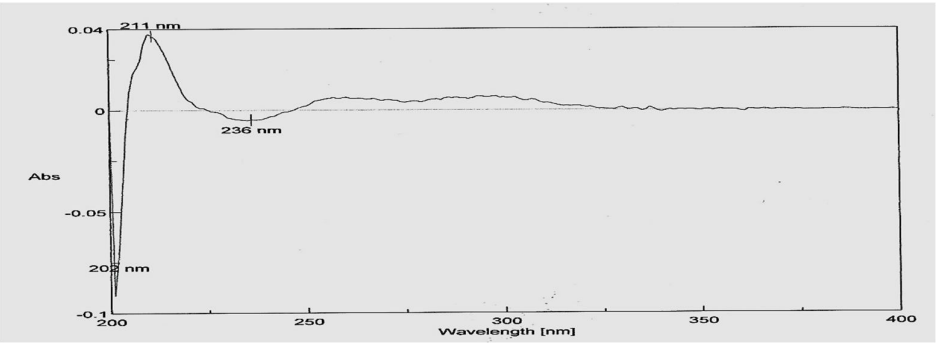


Figure (3.2.1.2) First derivative. UV Spectrum of Atorvastatin calcium in 50% methanol

3.2.1.3 Determination of absorptivity

Molar absorptivity, also known as the molar extinction coefficient, is a measure of how well a chemical species absorbs a given wavelength of light. It allows you to make comparisons between compounds without taking into account differences in concentration or solution length during measurements. It is commonly used in chemistry and should not be confused with the extinction coefficient, which is used more often in physics. The standard units for molar absorptivity are liters per mole centimeter (L mol^{-1} cm^{-1}), (Joanna, 2012).

Table (3.2.1.1) Determination of molar absorptivity

Concentration in µg/ml	Absorbance at 236nm	ε Molar absorptivity
16	0.246	153.75
18	0.278	153.44
20	0.309	154.50
22	0.343	155.91
24	0.373	155.41

3.2.1.4 Assay of tablet formulation

Validation of an analytical method is the process to establish that the performance characteristics of the developed method meet the requirements of the intended analytical application. The UV method was validated in the term of linearity, accuracy, precision, LOD, LOQ and sensitivity. The method was capable to analyze the active ingredient in atorvastatin calcium without interference of excipients.

Table (3.2.1.2) Assay result of Atorvastatin calcium in tablets

Drug	Batch No	Label claim mg/Tablet	Amount * found	%Recovery	%RSD
Phizer Egypt	2205	20	20.03	100.15	0.40
Amistatin Sudan	TOV001	20	19.95	99.75	0.45
Atorva Zydus India	GX2340	20	20.05	100.25	0.025
Avas India	FH0092	20	20.38	101.90	0.06
Atorvasal Sudan	4131	20	20.21	101.05	0.07

*Average of three determination

3.2.1.5. Recovery Studies (Accuracy)

This parameter is performed to determine the closeness of test results with that true value which is expressed as % recovery. The studies were performed at five level (80, 90,100,110 and120%) and the recovery of atorvastatin calcium calculated. Table (3.2.1.2)

Table (3.2.1.3) Recovery data of Atorvastatin calcium

Level of method%	Amount found	Recovery%	RSD %
80	79.61	99.51	
80	79.61	99.51	
80	79.29	99.11	0.23
90	89.96	99.96	
90	89.64	99.60	
90	90.30	100.33	0.36
100	100.00	100.00	
100	99.67	99.67	
100	100.32	100.32	0.32
110	109.70	99.73	
110	111.65	101.50	
110	112.29	102.0	1.21
120	120.38	100.32	
120	120.71	100.6	
120	120.71	100.6	0.62

3.2.1.6 Precision

The precision (system method) of proposed method was evaluated the assay were performed by repeatability (intraday) and intermediate reported as RSD%. Tabulated in Table (3.2.1.4).

3.2.1.7 Intermediate precision

Intermediate precision of the assay was determined for 100% concentration with six injections, three analyst Table (3.2.1.5).

Table (3.2.1.4) repeatability precision of system method

Run	Absorbance at 236nm
1	0.308
2	0.305
3	0.307
4	0.309
5	0.308
6	0.308
Mean	0.3075
RSD%	0.44

Table (3.2.1.5) intermediate precision data of Atorvastatin calcium

Run	ABS analyst X	ABS analyst Y	ABS analyst Z
1	0.306	0.311	0.307
2	0.310	0.307	0.308
3	0.307	0.310	0.310
Mean	0.308	0.309	0.308
RSD%	0.675	0.673	0.495

3.2.1.8 Determination of linearity and range

Linearity was evaluated through a linear regression analysis. The linearity for atorvastatin calcium (80 -120%) were determined in term of correlation coefficient, .which is equal to 0.9982.

Table (3.2.1.6) Standard curve of Atorvastatin calcium

Method level	Actual amount	RSD%
80	79.35	0.23
90	91.18	0.36
100	99.99	0.32
110	109.83	1.21
120	118.73	0.62

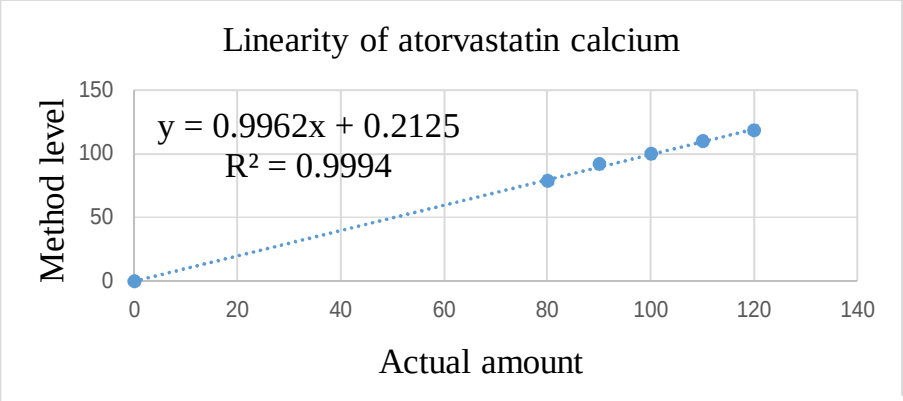


Figure (3.2.1.3.) Calibration curve of Atorvastatin Calcium ID_{236}

3.2.1.9 Limit of detection and limit of Quantitation

The limit of detection LOD and limit of quantitation LOQ for atorvastatin calcium is determined from the recession equation graph of ATC, LOD = 0.511µg/ml and LOQ =1.5487µg/ml.

3.2.1.10 Sensitivity

The sensitivity depends upon the experimental condition. The maximum sensitivity of which a method is capable is expressed in term of detection limit. In this manner very small amounts of the constituent must be obtained by small changes in the concentration.

Sensitivity of proposed methods is determined by calculating Sandell's sensitivity The Sandell's sensitivity is the concentration of the analyte (in µg mL-1) which will give an absorbance of 0.001 in a cell of path length 1 cm and is expressed as µg cm^2.((Divya *et a*l.,2012).

Table (3.2.1.7) sensitivity data for atorvastatin calcium

Concentration µg/ml	Absorbance at 236nm	Sandell's sensitivity
16	0.246	0.0650
18	0.278	0.0647
20	0.309	0.0647
22	0.343	0.0641
24	0.373	0.0643

3.2.1.11 Force degradation study

Forced degradation is a degradation of new drug substance and drug product at conditions more severe than accelerated conditions. It is required to demonstrate specificity Forced degradation studies show the chemical behavior of the molecule (ICH, 2003).

Table (3.2. 1.8) Degradation results of atorvastatin calcium

S.no	Absorbance	Assay	%Degradation
Control	0.330	99.86	0.0
Acidic	0.314	95.02	4.84
Oxidation	0.335	99.34	0.51
Thermal	0.305	92.3	7.60
Alkali	0.317	95.93	3.93

Table (3.2.1.9) Validation parameters of Atorvastatin calcium absorption at ID_{236}

Parameters	Results
Linear range	16 to 24µg/ml
Molar absorptivity Ɛ	154.60
Regression equation	Y=0.9741x+2.406
Slope	0.9741
Intercept	2.406
Sandell's sensitivity	0.0646
LOD	0.511 µg/ml
LOQ	1.548 µg/ml
r^2 value	0.9972

A linear correlation was found between absorbance and concentration of drug. Optical parameters such as molar absorptivity, Beer's law limit, and the proposed methods do not require any pretreatment of the drugs and tedious extraction procedure prior to their analysis. The newly developed methods are sensitive enough to enable quantification of the drug at low concentrations. These advantages encourage the application of the proposed methods in routine quality control analysis of atorvastatin calcium

A simple, sensitive and accurate spectrophotometric method has been developed for determination of atorvastatin calcium in raw material and tablets, using UV derivative. The solvent used was 50% methanol. ID236nm was elected as UV detection. All the calibration curve shows a linear relationship between the absorbance and concentration and coefficient correlation was found to be 0.9972.The regression of the curve was Y =0.9741x+2.406,.RSD 0.44% value is below 2.0 for repeatability precision which indicated that the method is highly précised. The percentage recovery value 99.82% indicates the accuracy of the method and absence of interference of the excipients present in the formulation, so the method is fit for analysis of atorvastatin in bulk and pharmaceutical formulation. Linearity relationships of the peak area over the mentioned concentration range of losartan potassium, were obtained. All parameters values are within linearity criteria which state $r \geq 0.99$.

3.2.2. Chemometric analysis:

Chemometrics is the chemical discipline that uses mathematical and statistical method to solve both descriptive and predictive problems in experimental natural sciences, especially in chemistry. In descriptive applications, properties of chemical systems are modeled with the intent of learning the underlying relationships and structure of the system (i.e., model understanding and identification). In predictive applications,

properties of chemical systems are modeled with the intent of predicting new properties or behavior of interest. In both cases, the datasets can be small but are often very large and highly complex, involving hundreds to thousands of variables, and hundreds to thousands of cases or observations. Chemometrics uses mathematical and statistical methods to improve understanding of chemical information, to select optimal experimental procedures, to provide maximum relevant chemical information by analyzing chemical data, and to obtain knowledge about chemical systems (Geladi and Esbensen, 2005).

Chemometric namely the factorial design approach was used to optimize the mobile phase composition in this work in development and validation methods and the resulting efficiency of separation, linearity, accuracy, LOD, LOQ and robustness, also comparison study with compendial HPLC method (Malinowski and Howery, 2002).

3.2.3. Development and Validation of High performance Liquid Chromatography Methods for determination of atorvastatin, method No 1

The traditional approach to HPLC optimization is to perform an experiment by trial and error or by change one control variable at time; such method can frequently require a very large number of experiments to identify the optimal condition. Recently computer assessed to HPLC separation has addressed the problem using factorial design strategies.

In this work a three factors with two level was applied to predict the retention behavior of Atorvastatin calcium and optimize their isocratic elution using acetonitrile as organic modifier and buffer as mobile phase. An eight- run ,2^3 factorial design of three factors at two level was set up to

standardize the spectrographic condition which are likely to be employed .Percentage of acetonitrile in the organic phase (X1), proportion of orthophosphoric acid 0.1 %(X2) and flow rate (X3) as per 2^3 factorial design are represented in the table Factors and their corresponding levels as per 2^3 factorial design.

X1, X2 and X3 are independent factors whereas Y1, Y2 and Y3 are dependent factors.

Factors and their corresponding levels as per 2^3 factorial design tabulated in Table (2.3.5.1) and Table (2.3.5.2).

3.2.3.1 Regression analysis

Most statistical software packages used are Minitab17, SPSS 22, Prism 6.0 or even Microsoft Excel are used to analyze data.

The targeted response parameters were statistically analyzed by appalling linear regression model, the significant terms ($P \leq 0.05$). The results of analysis of variance models generated by general linear model. The obtained adjusted R^2 were within acceptable limit of $R^2 \geq 0.80$, indicating that the experimental data were a good fit to the equation (Milkica *et al.*, 2013), (Wayne .W, 1991).

$$Y = ß_0+\beta_1 X_1 +\beta_2X_2+\beta_3X_3+ \beta_4X_1X_2+ \beta_5X_2X_3+ \beta_6X_1X_3+ \beta_7X_1X_2X_3.$$

Where, Y is the level of the measured response, ß0 is the intercept, β_1 to β_7 are the regression coefficients, X_1, X_2 and X_3 stand for the main effects, $X_1 X_2$, $X_2 X_3$ and $X_1 X_3$ are the two- way interaction between the main effects and $X_1 X_2 X_3$ is the three-way interact between the main effect. All the factors (X1, X2 and X3) were found to be significant (p value less than ≤ 0.05 for response of retention time (Y1).

Response models with p –value and statistical parameters, R^2 =0.977, Adjusted R^2= 0.9473 and SD= 0.16221.

Buffer solution in the mobile phase so the positive sign indicate direct correlation between response and factor, while a minus shows decreasing response with increasing factor. P –Values for all factors, X1(0.05), X2 (0.004) and X3 (0.001).

Table (3.2.3.1) Method number 1 chromatographic conditions employed as per 2^3 factorial design.

Exp No	Response			Response		
	X1	X2	X3	Y1	Y2	Y3
1	65	35	1.0	5.52	1.44	7508
2	70	35	1.0	5.10	1.54	6321
3	65	40	1.0	6.28	1.27	8760
4	70	40	1.0	6.08	1.48	7367
5	65	35	1.5	3.78	1.36	4447
6	70	35	1.5	3.56	1.58	4556
7	65	40	1.5	4.35	1.46	5087
8	70	40	1.5	3.92	1.36	4808

Y1 retention time, Y2 asymmetry 5%, Y3 Peak height

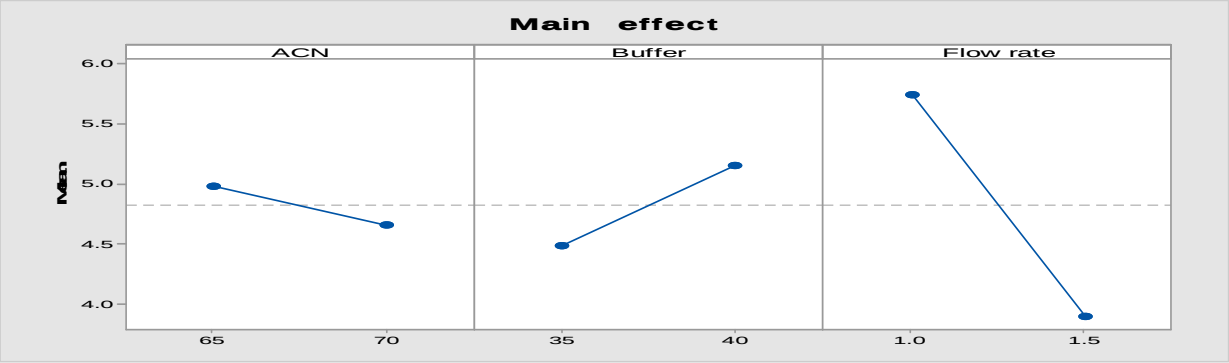
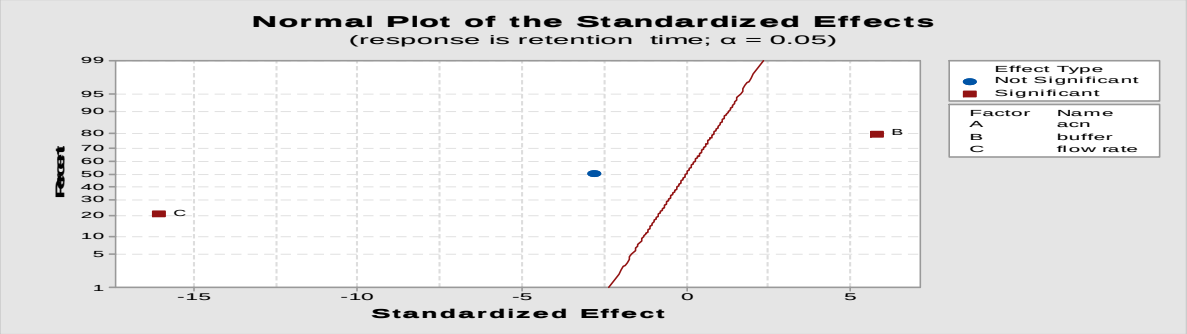


Figure (3.2.3.1) the main effect of retention time factors at p value 0.05



Figure(3.2.3.2) Normal plot of the standard effect retention time

In this work HPLC method for determination of Atorvastatin calcium in tablets and their degradation products was developed and validated. The optimization of the method was done by selected of suitable solvents such as methanol and acetonitrile, different columns C8, C18, detection wavelength and analyte concentration. The detection wavelength of 246nm was selected after scanning the standard solution over range 200-400nm by using UV detector.

3.2.3.2 Linearity

The linearity study was performed and the correlation coefficient of atorvastatin calcium was found to be 0.9998.

Table (3.2.3.3) Calibration curve of Atorvastatin calcium

Con(µg/ml)	Peak area at 246nm			
6.4	Inj1	Inj2	Inj3	Mean
	183.4	183.19	183.76	183.45
7.2	202.40	201.11	203.47	203.33
8.0	226.16	224.83	226.76	225.92
8.8	251.84	250.17	249.48	250.50
9.6	275.85	274.16	275.79	275.27
Atorvastatin calcium			Slope	28.503
			Intercept	0.2782
			r^2	0.9998
			Std.Erro	0.065

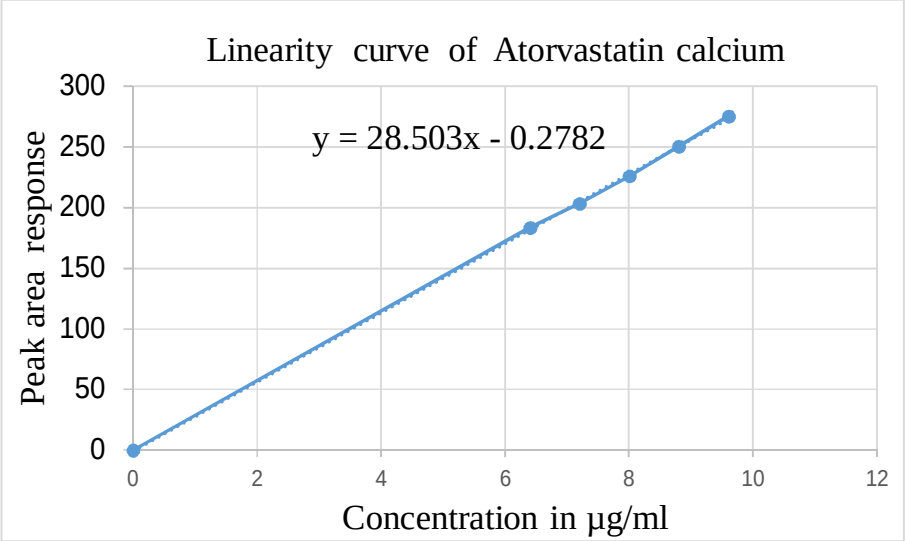


Figure (3.2.3.3) Linearity curve of Atorvastatin calcium

The accuracy of the method was assessed by determination of recovery for three concentration covering the range of the method. The amount of atorvastatin calcium was recovered in the presence of placebo interference, was calculated. The mean recovery of Atorvastatin was 100.49% which is satisfactory and result shown in Table (3.2.3.3).

Table (3.2.3.3) Recovery for Atorvastatin calcium

Sample level	Actual amount added mg	Amount recovery	% Recovery
80	20	20.15	100.70
80	20	20.10	100.50
80	20	20.18	100.90
100	20	19.87	99.37
100	20	20.37	101.83
100	20	19.76	98.80
120	20	20.20	101.00
120	20	20.07	100.35
120	20	20.19	100.98
		Mean ±SD	100.49±0.86

3.2.3.3 System suitability

The obtained results of system suitability showed that the HPLC system is capable of providing high recovery Table (3.2.3.4).

Table (3.2.3.4) System suitability data for Atorvastatin

No	Peak area reproducibility at 246nm	Retention time-min	Asymmetry 5 %	Theoretical plate
1	249.9690	4.983	1.25	7739
2	251.0010	4.983	1.25	6115
3	252.7550	4.983	1.25	6115
4	251.2100	4.983	1.25	6115
5	255.1990	5.00	1.25	6156
6	254.4840	5.00	1.25	7791
Mean	252.4360	4.988	1.25	6671.80
RSD	0.822%	0.175%		
Limit	T 2%	NMT 1%	NMT2%	NLT 2000

3.2.3.4 Selectivity

The poropsed method has ability to separate the analyte in the presence of comoponents that may be expected to be present such as impurites, degradation products and excipients.Specificity measures only the desired component without interference from other species that might be present.(Ahuja and Rasmussen,2011).

For stress testing under different conditions, the major degradation under acidic condition up to 80.42% Table (3.2.3.6); under alkaline condition atorvastatin calcium showed no degradation was observed .The drug was approximately 4.36% degradated under oxidizing condition Figure (3.2.3.6) ; the drug was degradated 0.05% under thermal condition and 4.16 % degradation occurred under UV radiation at 254nm .

Table (3.2.3.5) Degradation of stress testing of Atorvastatin calcium

S.NO	Condition	% Assay of atorvastatin calcium	% degradation	
			Single maximum	Total
1	No stress treatment(control sample)	99.89	Nil	Nil
2	Acid	19.567	55.128	80.42
3	Alkali	100.4	Nil	Nil
4	H_2O_2	95.55	3.19	4.36
5	UV	95.70	4.16	4.16
6	Thermal	99.84	0.06	0.06

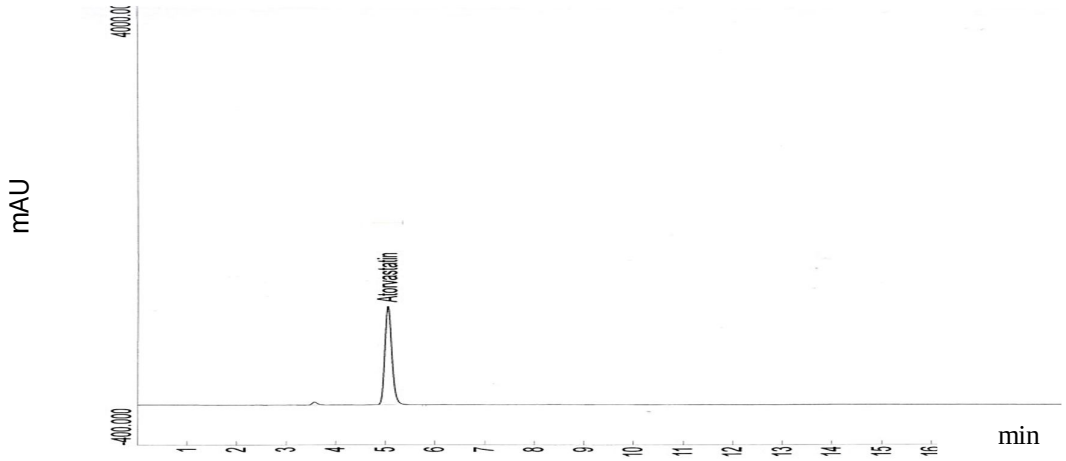


Figure (3.2.3.4) HPLC Chromatogram of Atorvastatin calcium (standard)

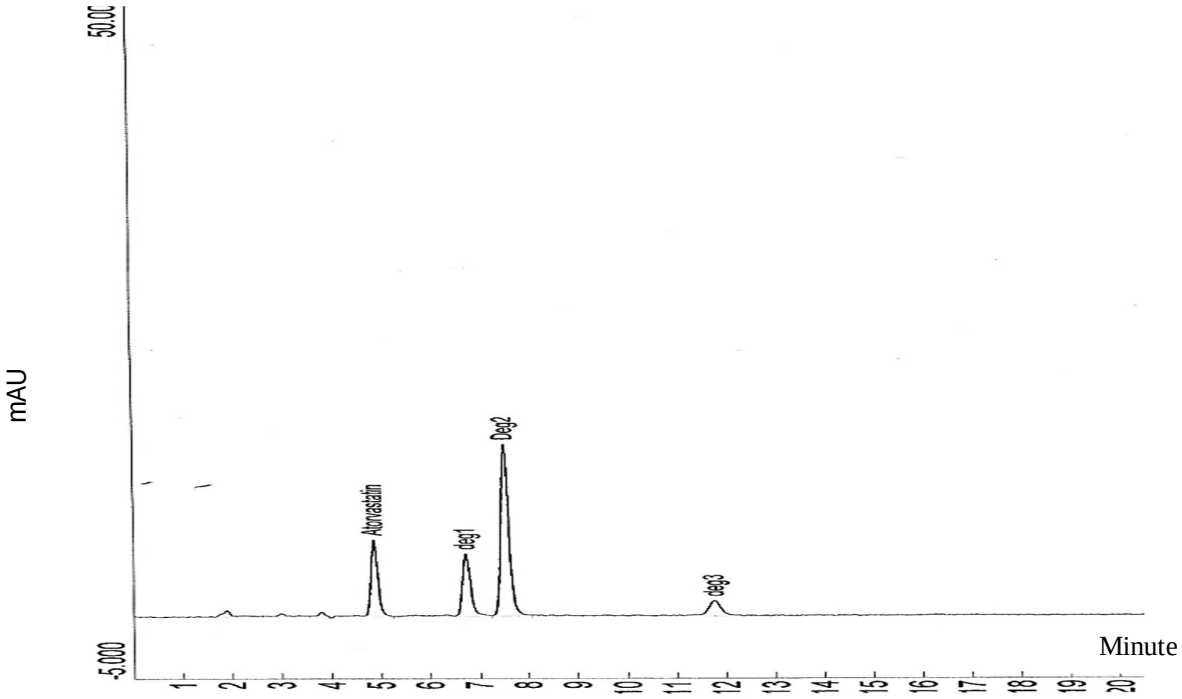


Figure (3.2.3.5) HPLC Chromotogram of acid Degradatin of Atorvastatin

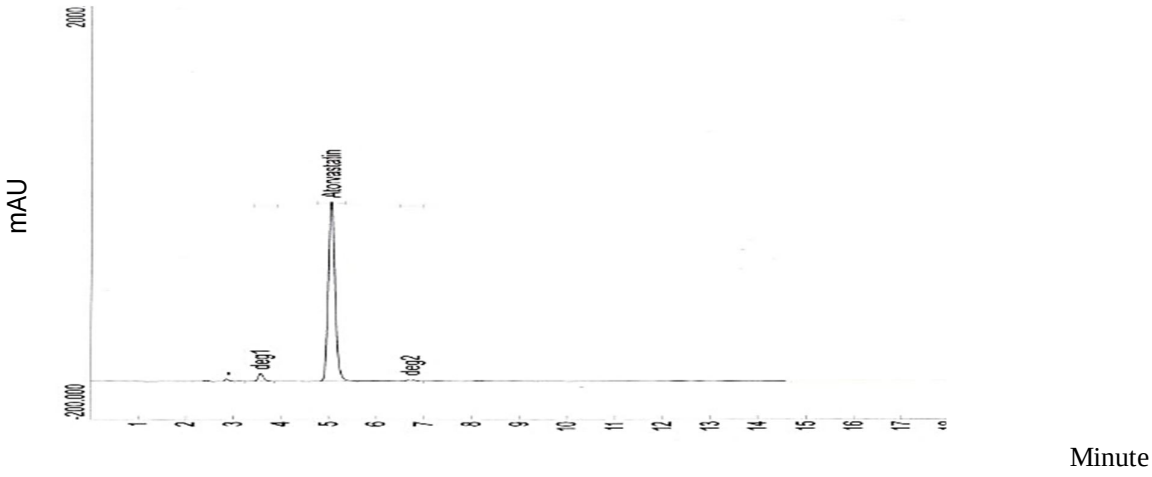


Figure (3.2.3.6) HPLC Chromatogram of peroxide effect of Atorvastatin

Under experimental conditions described, the recovery indicated that the method was suitable for its intended purpose .The specificity of the method was determined by checking for interference with the drug from placebo component and also specificity evaluated by checking the peak

purity of the analyte during the stress testing study. Indicated that no interference with the analyte peak result. Which tabulated in Table (3.2.3.6) and Figure (3. 2.3.5).

3.2.3.5 Precision

Precision of the method were done in the % RSD for repeatability was found 0.82%, and intermediate precision 0.29.From the data obtained the method was found to be precise, the accuracy of the method was assessed by determination of recovery for three concentration covering the range of the method

The objective to study precision was to establish that promoted RP-HPLC is accurate for analyzing atorvastatin calcium in pharmaceutical formulation as well as bulk forms.

The precision (system method) of the proposed method was evaluated by carrying out six independent assays of test sample. RSD 0.484% of six assay value obtained was calculated .The intermediate precision was carried out by analyzing the sample in different days.

Table (3.2.3.6) Intra-day precision of Atorvastatin calcium

Repeatability precision		Retention time	Peak area at 246nm	Assay
Sample	Concentration			
Atorvastatin	8µg/ml	4.783	233.12	99.96
		4.883	233.95	100.31
		4.883	234.99	100.76
		4.883	233.15	99.97
		4.883	232.37	99.63
		4.866	231.76	99.40
RSD		0.82	0.49	0.484

Table (3.2.3.7) Inter – day precision of atorvastatin calcium

Intermediate precision		Inject	Peak area at 246nm	%Assay
Sample	Concentration			
Atorvastatin	8µg/ml	1	243.78	100.5
		2	242.24	99.86
		3	243.1	100.22
		4	241.93	99.73
		5	242.20	99.84
		6	242.14	99.82
RSD			0.29	0.29

3.2.3.6 Robustness (Praveen and Yusra, 2012).

The robustness of the method was assessed by changing of the wavelength, flow rate and mobile phase composition. System suitability data were found to be satisfactory during variation of analytical conditions.

Robustness of the method was determined by small deliberate changes in flow rate, and wavelength. The content of the drug was not adversely affected by these changes as evident from the low value of relative standard deviation indicating that the method was rugged and robust.

Table (3.2.3.8) Robustness change of wavelength

Injection	Change in wavelength±2.0nm	% Estimation of Atorvastatin
1	244nm	99.40
2	246nm	100.79
3	248nm	99.60
Mean		99.93
RSD%		0.73

Table (3.2.3.9) Change of flow rate

Flow rate(ml/min)			Peak area	RSD %	t_R	% RSD
Original	Used	Level				
1.0	0.9	-0.1	354.384	0.36	5.533	0.00
	1.0	0	326.4726	0.47	5.00	0.00
	1.10	+0.1	283.440	0.75	4.410	0.21

Table (3.2.3.10) Change of mobile phase composition

Mobile phase Composition			Peak area	RSD%	t_R	% RSD
Original	Used	Level				
66:34	65:35	-1.0	325.748	0.11	5.00	0.00
	66:34	0.0	331.930	0.50	4.910	0.188
	67:33	+1.0	331.010	1.62	4.883	0.00

3.2.3.7 Stability of system

The standard and sample solution of an atorvastatin have reasonably good stability over 24 hours.

Table (3.2.3.11) Solution stability

Time	Peak area of standard solution at 246nm	Peak area of sample solution at 246nm	% estimation
Zero time	260.49	266.20	99.99
24 hours	255.8	256.14	100.80
48	245.6	248.11	100.00
Mean			100.30
RSD			0.38%

Table (3.2.3.12) Analytical results of Atorvastatin calcium tablet 20mg

Drug	n	Amount claimed(mg/tablet)	Amount found (mg/tablet)	Recovery	RSD
Atorvastatin	6	20	20.14	100.40	0.40

Table (3.2.3.13) Summary of validation parameters for method No1

Parameters	Observations	Accepted criteria
Specificity	No interference was found w.r.t excipients	Resolution ≥1.25
Linearity r^2	0.9998	$r^2 \geq 0.99$
Range	80 -120% of test concentration	50 -150%
Precision(RSD) a)Repeatability(n=6) (system precision) b)Intermediate precision (inter-day analyst) (n=6)	 0.484 0.290	RSD ≤ 2.0
Solution Stability	0.38	RSD ≤ 2.0
Accuracy(% recovery)	100.49±0.86	
Stability in analytical solution	Stable	24 hours
Stress degradation	The peaks were pure without any interference	Resolution ≥1.25
Robustness(overall RSD) Change of wavelength 244nm 248nm Change in flow rate 0.9ml/min 1.0ml/min 1.1 ml/min Change in mobile phase composition 65:35 66:34 67:33	 0.730 0.36 0.47 0.75 0.112 0.50 1.62	 RSD ≤ 2.0

Assay value after 24 hours indicate that the method was accurate and precise. No interference from placebo was observed at retention time of the drug peak, atorvastatin calcium peak is pure and don't have any co eluting peak there for it is conducted that the method is specific.

RSD meet the acceptance criteria, it conducted that sample is stable in analytical solution for at least 48 hours.

The assay value for marketed formulation was found to be within the limits, as listed in Table (3.2.12), the low RSD value indicated the suitability of the method for routine analysis of Atorvastatin in pharmaceutical dosage form.

3.2.4 Method validation No 2

3.2.4.1 Optimization of the method

The experimental conditions should be optimized to get desired separations and sensitivity after getting appropriate separation , stability indicating assay experimental conditions will be achieved through planned/systemic examination on parameters including pH (if ionic), mobile phase components and ratio, gradient or isocratic ,flow rate temperature , sample amounts , injection volume and diluent solvent type.

Table (3.2.4.1) Method number 2 chromatographic conditions employed as per 2^3 factorial design

Exp No	Factors			Response		
	X1	X2	X3	Y1	Y2	Y3
1	60	30	1.0	4.10	1.29	69.30
2	65	30	1.0	4.616	1.13	66.140
3	60	35	1.0	5.833	1.22	53.953
4	65	35	1.0	5.20	1.11	60.366
5	60	30	1.3	3.133	1.17	62.70
6	65	30	1.3	3.566	1.33	60.181
7	60	35	1.3	4.60	125	49.184
8	65	35	1.3	3.983	1.14	54.743

3.2.4.2 Method development

To develop an efficient, rapid and simple stability indicating HPLC method for the assay of atorvastatin calcium, preliminary tests were conducted to select the suitable and optimum conditions HPLC parameters, such as detection wavelength, ideal mobile phase and right composition, flow rate and column packing material, particle size were carefully studied, the HPLC parameters were finally chosen based on the system suitability requirement.

3.2.4.3 Statistical analysis

In this work a three factors with two levels was applied to predict the retention behavior of Atorvastatin calcium and optimize their isocratic elution using acetonitrile as organic modifier, formic acid and Triethyl amine as mobile phase.

Response models with p –value and statistical parameters, R^2 =0.9995, Adjusted R^2=0.996 and SD=0.0526.

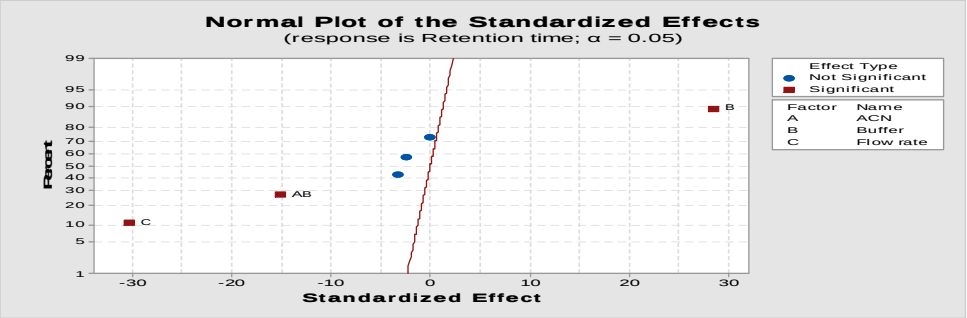


Figure (3.2.4.1.) standardized effect for retention time

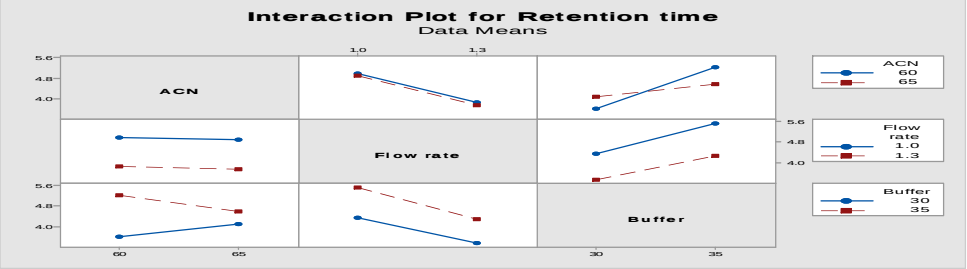


Figure (3.2.4.2.) Interaction plot for retention time

Mobile phase composition, for example, is the most powerful way of optimizing selectivity whereas temperature has a minor effect and would only achieve small selectivity changes. PH will only significantly affect the retention of weak acids and bases. The optimized HPLC method was validated in accordance with the International Conference on Harmonization guideline (ICH; 2005). Parameters such as system suitability, selectivity, sensitivity linearity range, accuracy, specificity and robustness were validated.

3.2.4.4 System suitability

Table (3.2.4.2) system suitability parameters

No	Peak area reproducibility at 246nm	Retention time-min	Asym 5 %	Theoretical plate
1	390.0140	4.950	1.38	6033.00
2	390.0690	5.00	1.38	6049.00
3	392.1205	4.983	1.36	6115.00
4	396.5180	4.966	1.38	6074.00
5	395.4130	4.983	1.38	6115.00
6	397.4860	5.00	1.36	6156.00
Mean	393.468	4.980	1.386	6090.33
RSD	0.8198%	0.392%	0.752%	0.762

3.2.4.5 Selectivity

Generally refers to the method that produces a response for the single analyte only.

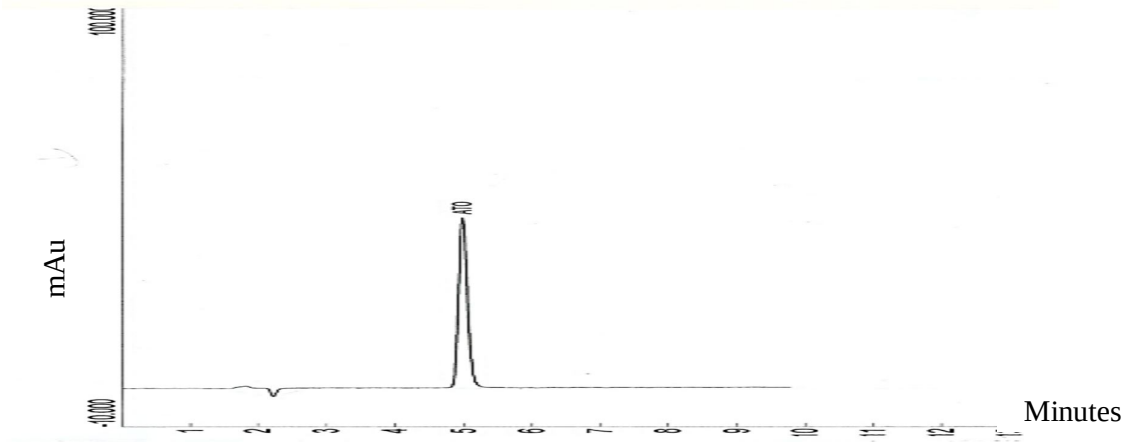


Figure (3.2.4.3) HPLC chromatogram of Atorvastatin reference standard

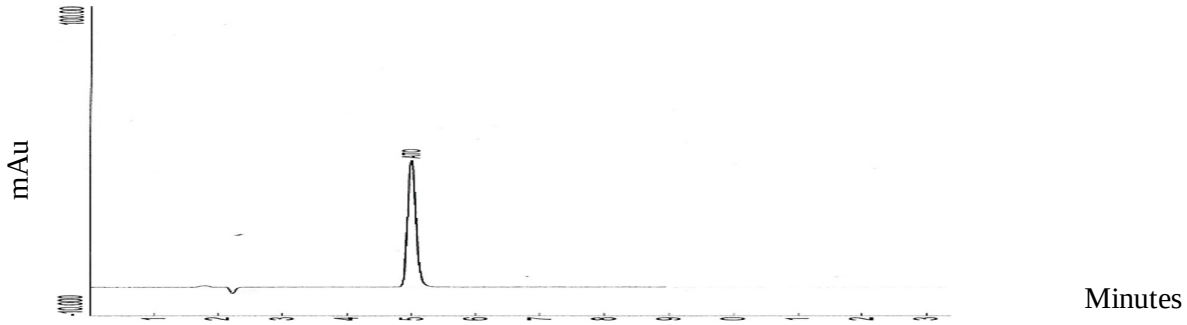


Figure (3.2.4.4) HPLC chromatogram of Atorvastatin tablet sample

3.2.4.6 Sensitivity

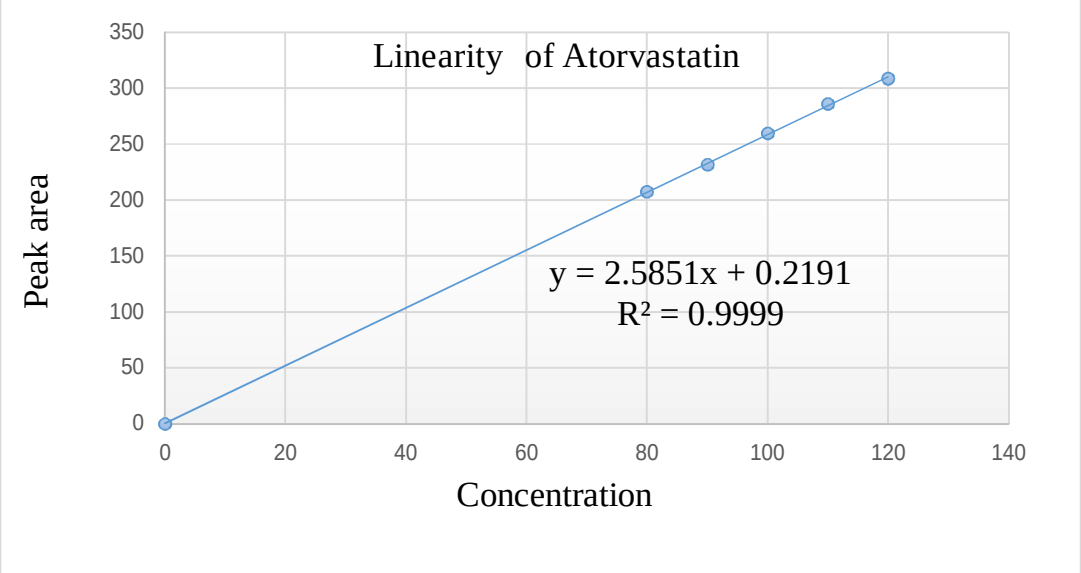
The sensitivity of HPLC proposed method was investigated through the measurement of the limit of detection (LOD) and limit of quantitation (LOQ) at a signal to noise of 3.3 and 10 respectively. It was achieved from the linearity equation. The LOD and LOQ were found to be 0.1.77µg/ml and 5.352µg/ml, these values suggest that the developed method was sensitive to quantify atorvastatin calcium.

3.2.4.7 Linearity

. The response should be directly proportional to the concentrations of the analytes; a linear regression equation applied to the results should have an intercept not significantly different from 0. If a significant non zero intercept is obtained, it should be demonstrated that this has no effect on the accuracy of the method.

Table (3.2.4.3) linearity of Atorvastatin Calcium

Level method%	Peak area at 246nm	Actual	RSD%
80	208.85	80.39	
80	205.67	79.17	0.82
80	208.31	80.18	
90	233.19	89.80	
90	231.40	89.00	0.45
90	230.82	89.29	
100	259.57	99.92	
100	258.89	99.65	0.39
100	260.88	100.42	
110	287.26	110.57	
110	287.63	110.72	0.86
110	283.18	109.00	
120	309.55	119.16	
120	307.76	118.50	0.46
120	308.67	119.00	



Figure(3.2.4.5) Linearity of Atorvastatin calcium

3.2.4. 8 Precision and accuracy

The precision and accuracy of the method was determined by intra-day (repeatability) and inter –day (intermediate) assay and was expressed as relative standard deviation and recovery percentage, respectively.

Table (3.2.4.4) Repeatability data

Injection No	Retention time	Peak area at 246nm
1	4.866	264.2790
2	4.850	265.5570
3	4.816	265.0810
4	4.833	263.3580
5	4.850	264.7130
6	4.816	262.9915
Mean	4.860	264.3299
RSD	0.867%	0.38%

Table (3.2.4.5) Intermediate precision data

N^*=3	Peak area analyst X	Peak area analyst Y	Peak area analyst Z
Mean	263.50	262.52	262.37
RSD	0.0042%	0.91%	0.34%

* Average of three injection

3.2.4.9 Specificity

In order to estimate the specificity of the proposed method, stress degradation studies were performed using different ICH prescribed stress condition such an acidic, alkali, oxidation, thermal and photolytic stresses. Under acid degradation Atorvastatin three products was produced, Table (3.2.4.6) and Figure (3.2.4.6), therefore the method was successfully separated the degradation products from analyte peak so the method was

specific for determination atorvastatin calcium in the presence of degradation products.

Table (3.2.4. 6) Acid degradation of atorvastatin calcium

Component	Retention time	Total plate	Asym 5%	Resolution	Degradation %
Atorvastatin calcium	5.05	4203	1.44	0.0	19.25
Decompose1	7.283	7347	1.50	5.826	13.65
Decompose2	8.183	7903	1.50	2.16	59.41
Decompose3	13.00	9337	0.80	9.031	7.65

Table (3.2.4.7) Stress testing of atorvastatin calcium

.S.NO	Condition	% Assay of atorvastatin calcium	% degradation
1	No stress treatment(control sample)	99.96	Nil
2	Acid	19.25	80.71
3	Alkali	100.4	Nil
4	H_2O_2	95.55	4.41
5	UV	95.70	4.26
6	Thermal	99.30	0.66

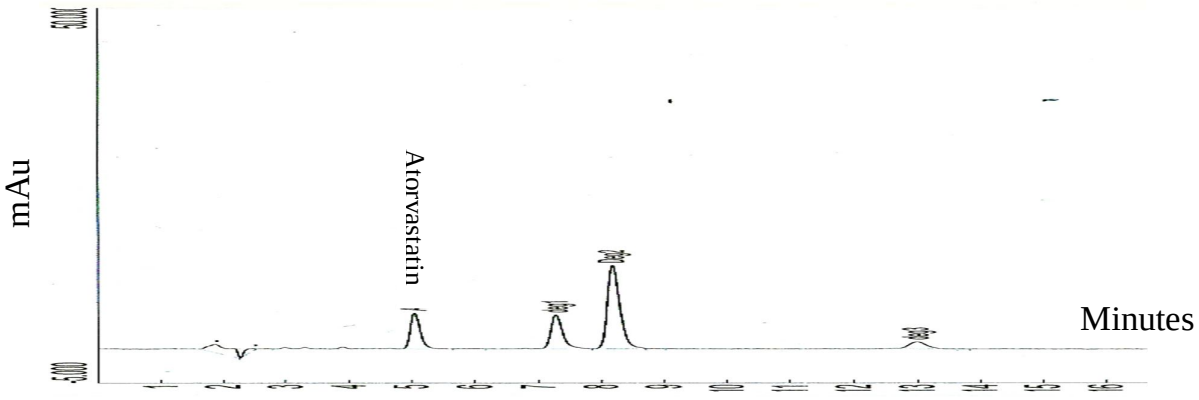


Figure (3.2.4.6) Acid degradation of Atorvastatin calcium

Table (3.2.4.8) Assay of Atorvastatin calcium tablet 40 mg

Drug	n	Amount claimed (mg/tablet)	Amount found (mg/tablet)	Recovery	RSD
Atorvastatin	6	40	39.90	99.77	0.59

3.4.2.10 Robustness

According to ICH robustness is the measure of the capacity of a method to remain unaffected by small, variations in parameters used in the method.

Table (3.2.4.9) method robustness with variable flow rate

Flow rate(ml/min)			Peak area	RSD%	t_R	% RSD
Original	Used	Level				
1.0	0.9	-0.1	409.9690 410.3770 410.3870	0.058	5.483 5.483 5.483	0.00
	1.0	0	390.0140 390.0690 392.1205	0.307	4.950 5.000 4.983	0.51
	1.10	+0.1	307.0270 313.4640 310.0590	1.038	4.166 4.133 4.166	0.46

Table (3.2.4.10) method robustness with variable mobile phase composition

Mobile phase composition			Peak area	RSD%	t_R	% RSD
Original	Used	Level				
65:35	64:35	-0.1	340.4580 341.9215 345.6550	0.78	4.883 4.900 4.900	0.20
	65:35	0	373.450 374.1480 370.4430	0.53	4.916 4.933 4.883	0.52
	66:35	+0.1	345.7610 347.5705 347.9690	0.34	4.883 4.833 4.833	0.00

Table (3.2.4.11) Summary of validation parameters method number 1

Parameters	Observations	Accepted criteria
Specificity	No interference was found w.r.t excipients	Resolution ≥1.25
Linearity r^2	0.999	$r^2 \geq 0.99$
Range	80 -120% of test concentration	50-150%
Precision(RSD) a)Repeatability(n=6) (system precision) b)Intermediate precision (inter-day analyst) (n=6)	0.29 0.822	 RSD ≤2.0
Solution Stability	0.38	RSD ≤2.0
Accuracy(% recovery)	100.35±0.66	98-102%
Stability in analytical solution	Stable	24 hours
Stress degradation	The peaks were pure without any interference	No interference
Robustness(overall RSD) a) Change in flow rate b) Change in mobile phase composition	 0.484 0.510	 RSD ≤2.0

Table (3.2.4.12) comparison between two proposed method number 1 and 2 of determination atorvastatin.

method	LOD	LOQ	Adjusted R^2	R^2	Standard error	Retention Time
Method 1	0.337µg/ml	1.022µg/ml	0.977	0.9473	0.164	4.988 min
Method 2	1.773µg/ml	5.353µg/ml	0.9995	0.996	0.0526	5.01min

A 2^3 factorial design consisting of 3 factors at 2 levels was set up to standardize the chromatographic condition, the base 2 stands for the level of condition i.e, maximum and minimum and the power 3 stand for number of condition i.e buffer concentration, organic modifire like acetonitrile or flow rate of mobile phase.

A rapid, reversed-phase liquid chromatographic method was developed for the quantitative determination of Atorvastatin calcium, its degradation products. The chromatographic separation was achieved on RP-HPLC method utilized a 5µm particle Kromasil 10 column C18, (250mmx4.6mm) at ambient temprature , The mobile phase consisted of acetonitrile, formic acid 20m M and 0.1% triethyl amine(65:35v/v) ,the mobile phase was delivered isocratically at flow rate of 1.0 ml/min with UV detection at 246 nm.The calibration plots constructed using the optimized chromatographic conditions displayed good linearity relationship in the concentration range of 6.4-9.6µg/ml, (80-120%) with r=0.999.The method was validated for precision,accuracy, robustness and recovery.the minim detactable and minimum quantifiable amount were found to be 2.162 and 6.55 µg/ml, respectivily and method was found to be reproducible from statistical data generated.Atorvastatin calcium was eluted at 5.0min.

The most important factors that affect peak asymmetry are flow rate and temperature. The method, however, is robust with respect to peak asymmetry; this value was consistent <1.2 across all values of these parameters. Method suitability, therefore, will not be adversely affected with respect to peak asymmetry (Snyder, 1980).

Flow rate is the parameter that significantly affects peak efficiency. The method, however, is robust with respect to peak efficiency; this value was consistent >2000 across all values of these parameters. Method suitability, therefore, will not be adversely affected with respect to peak efficiency.

The robustness of the method was illustrated by change the flow rate of mobile phase and the composition of mobile phase, the proposed method remained unaffected by slight change in the analytical conditions Table (3.2.4.9) and Table (3.2.4.10).

Factorial design employed by using computerization software of the method development and validation are useful in analyze of pharmaceutical industry .In this work a simple and rapid methods has been developed to determination of atorvastatin in pharmaceutical formulation and in presence of degradation products. Linear model postulate an 2^3 was employed to estimate the model coefficients.

3.2.5 Development and Validation of an UV derivative spectro-photometric determination of losartan potassium

The aim of the present study was to investigate the validation and application of analytical methods for quality control of 50 mg losartan potassium pharmaceutical capsules, using first-derivative UV spectro-photometry.

The propose and validate a new procedure t determine Losartan potassium drug substance when it is as single active in tablet, based on UV derivative spectrophotometry, which is recommended for losartan potassium in tablet, but the recommended linear range of the method is very narrow (4- 6 µg/ml) and the result was not compared to standard method such as HPLC. The aim of this work was to develop an alternative analytical method that could be used for individual analysis of tablet and fulfilling the requirements of analytical quality necessary to be applied to the content uniformity test including by for finished pharmaceutical products, (Parra *et al*.,1993).

3.2.5.1 Method optimization

3.2.5.1.1 Solubility determination

Solubility of Losartan potassium was determined in different solvents. Losartan was found to be soluble in water and methanol.

3.2.5.1.2 Selection of detection wavelength

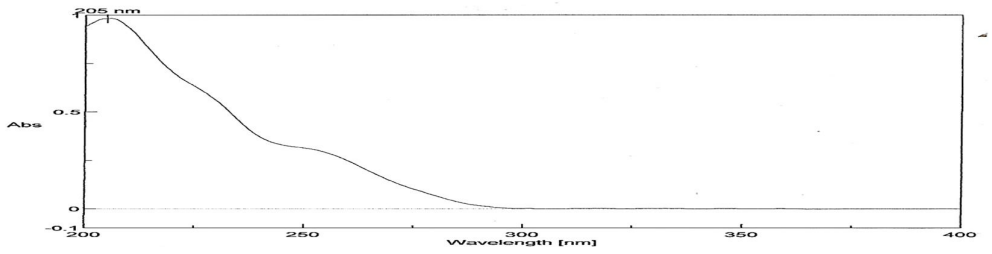


Figure (3.2.5.1.) Zero-order UV spectrum of losartan potassium

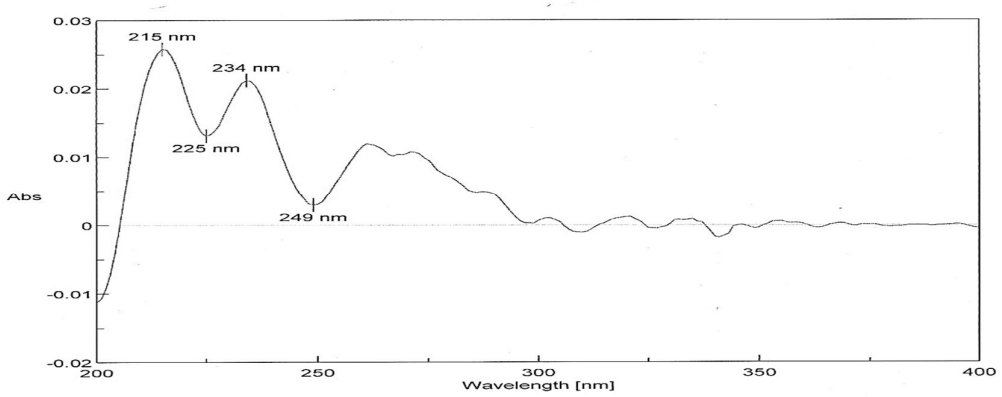


Figure (3.2.5.2) first – derivative UV spectrum of Losartan potassium

3.2.5.2 Quantitative analysis of losartan potassium tablets

The derivative spectrum of any order results from the differentiation of a zero order (basic) spectrum Figure (3.2.5.1) and Figure (3.2.5.2), the ID_{234} used for determination assay of pharmaceutical samples from different brand Table(3.2.5.1). This property is used to improve the accuracy of quantification of a narrow band component in the presence of a broad band component and to reduce error caused by scattering (Parra, *et al*, 1993).

Table (3.2.5.1) Assay of Losartan potassium in tablet dosage form

Drug	Batch No	Label claim mg/Tablet	Amount * found	%Recovery	%RSD
Losacar India	GP1933C	50	50.26	100.52	0.36
Amilosan Sudan	TNS001	50	49.64	99.28	0.49
Nilosaan Sudan	2NZ02	50	48.95	98.00	0.80
Cozal Sudan	4161	50	50.02	100.04	0.37
Zyltan India	Z10528	50	50.92	101.84	0.10

*Average of three determination

3.2.5.3 Sensitivity

Adherence to Beer's law was studied by measuring the absorbance values of solutions varying in drug concentration. The analytical

parameters such as molar absorptivity, Beer's law limits and Sandell's sensitivity values were calculated Table (3.2.5.2). Sensitivity of the proposed methods is determined by calculating Sandell's sensitivity (µg/cm2/0.001 Abs unit), which can be defined as smallest weight of substance that can be detected in column of unit cross-section, (Kanchan, 2014).

Table (3.2.5.2) Sensitivity data of Losartan potassium

Concentration µg/ml	ABS at 234nm	Sandell's sensitivity
4	0.220	0.0182
6	0.324	0.0185
8	0.423	0.0189
10	0.541	0.0185
12	0.637	0.0188
14	0.740	0.0189

3.2.5.4 Determination of molar Absorptivity

The molar absorptivity Table (3.2.5.3) is unique to whatever the absorbing species. Some molecules have enormous molar absorptivities in certain regions of the visible spectrum; these are often used as dyes because a small amount absorbs a great deal of light and thus the substance will appear very deeply colored (in the wavelength region where the dye doesn't absorb). Even if two substances absorb in the same wavelength region and have the same concentration, they may appear very different in terms of the depth of color, due to differences in their molar absorptivity. (Pace, 1995).

Table (3.2.5.3) Molar absorptivity of Losartan potassium

Concentration .µ g/ml	ABS	Molar absorptivity ε
4	0.2204	551.0
4	0.2204	
4	0.2197	
6	0.3239	539.8
6	0.3238	
6	0.3249	
8	0.4229	528.62
8	0.4238	
8	0.4234	
10	0.5405	541.60
10	0.5416	
10	0.5428	
12	0.6363	531.90
12	0.6383	
12	0.6368	
14	0.7409	529.21
14	0.7409	
14	0.7393	

3.2.5.5 Linearity

The calibration graphs Table (3.2.5.4) are described by the equation Y= a+bx, where Y= absorbance, a= intercept = slope and x = concentrating in µg/ml.

Table (3.2.5.4) Data of calibration curve of losartan potassium

Concentration µg/ml	ABS	%RDS
4	0.220	0.18
6	0.324	0.18
8	0.423	0.10
10	0.541	0.21
12	0.637	0.16
14	0.740	0.12

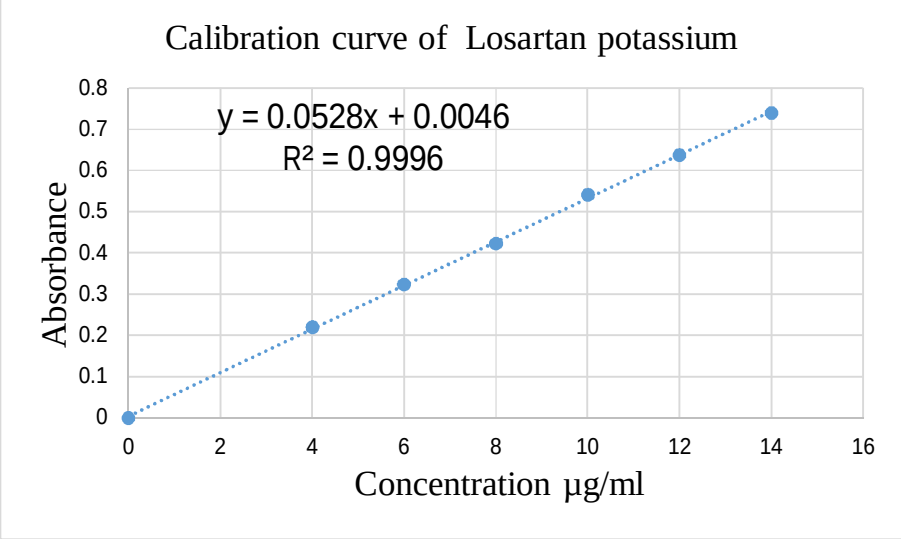


Figure (3.2.5.3). Plotting of data of calibration curve of losartan potassium vs ID_{234}

3.2.5.6 Accuracy

The accuracy of the methods established by analyzing the pure drugs at different levels within working limits and calculating the recovery Table (3.2.5.5), which show the good accuracy about 99.42% of the proposed method.

Table (3.2.5.5) Recovery studies (accuracy of the method)

Sample Added µg/ml	Amount of drug recovered µg/ml	Amount of drug	%recovery
1	4	4.07	101.75
2	6	5.98	99.70
3	8	7.84	98.00
4	10	9.98	99.80
5	12	11.80	98.33
6	14	13.85	98.93

3.2.5.7 Precision

Precision ascertained by calculating the relative standard deviation of replicate determinations on the same solution containing the drugs are presented in Table (3.2.5.6) and Table (3.2.5.7).

3.2.5.7.1 Repeatability precision

Table (3.2.5. 6) Repeatability of Losartan potassium

Repeatability precision		Run	Absorbance at 234nm	Assay	% RSD N=6
Sample	Concentration				
Losartan potassium	10µg/ml	1	0.5385	99.89	0.102
		2	0.5393	100.04	
		3	0.5399	100.15	
		4	0.5386	99.90	
		5	0.5397	100.11	
		6	0.5390	99.98	

3.2.5.7.2 Intermediate precision

Table (3.2.5.7) Data of inter- day intermediate precision

Intermediate precision		Run	Absorbance at 234nm	Assay	% RSD N=6
Sample	Concentration				
Losartan potassium	10µg/ml	1	0.5407	100.15	0.18
		2	05404	100.10	
		3	0.5406	100.13	
		4	0.5397	99.96	
		5	0.5380	99.65	
		6	0.5400	100.02	

Table (3.2.5.8) Validation parameters of Losartan potassium absorption at ID_{234}

Parameters	Results
Linear range	4 to 14µg/ml
Molar absorptivity	537.02
Sandell sensitivity	0.0186
Regression equation	Y =0.0528x+0.0046
Correlation coefficient(r^2)	0.9996
Slope	0.0528
Intercept	0.0046
LOD µg/ml	0.317
LOQ µg/ml	0.9615

The zero order spectra of pure losartan potassium was found to be difficult because it's shows a maximum absorption close to 202 nm an an ill-defined shoulder band featureless , extended from 225 to 240 nm, figure 2. This behavior precluded the analytical use zero order absorbance.

The calibration curve plot for the method was linear over the concentration range of 4 -14µg/ml. The determination of coefficients (r2) was found 0.9995, the method was found to be precise and the % RSD value for intra-day 0.102 and inter-day 0.18 respectively it was less than 1%.

Recovery percentage (99.42) was found to be good at each added concentration, indicating that the method was accurate. The result of assay showed that the amount of losartan potassium was good agreement with the label claim of formulation as indicated by % assay (99.93).

A simple, sensitive and accurate, low cost and require relatively inexpensive instrument, then it is good alternative to existing method for determination losartan potassium.

Pharmacopeias have not yet provided an official method for its quantification. Losartan potassium, the method employed is a first derivative spectroscopy a signal at 234 nm of the first derivative spectrum was found adequate for quantification. The linearity between signal 1D234 and concentration of Losartan potassium in the range of 4.00-14µg/ml in aqueous solutions presents a square correlation coefficient (r2) of 0.9995.the recovery studies confirm accuracy of proposed method and low values of standard deviation confirm precision of the method, the method is validated as per ICH guideline.

Simple spectrophotometric methods for the determination of losartan potassium have been developed and validated according to ICH guidelines. The proposed methods are accurate and precise as indicated by good recoveries of the drugs and low RSD values. All the analytical reagents are inexpensive, have excellent shelf life, and are available in any analytical laboratory. The methods could be used to monitor the content uniformity of tablets. The proposed methods could be applied for routine analysis and in quality control laboratories for quantitative determination

3.2.6 Development and Validation HPLC method for analysis of Losartan potassium method No. 1

BP described a potentiometric titration for assay of Losartan potassium. For impurities BP used a gradient RP-HPLC for Losartan potassium, some methods have been published for simultaneous determination of studied Angiotensin-II-drugs including (BP, 2015).

3.2.6.1 Optimization of Chromatographic Condition

The aim of this work, we report the development and validation of a stability indicating LC method for determination Losartan potassium in pharmaceutical dosage form. It separates drug components from degradation products under ICH suggested stress conditions (acid base hydrolysis, oxidation, photolysis and thermal stress).

The target in developing the stability indicating method is to achieve the resolution between the losartan potassium and its degradation products. Our goal of chromatographic method development was to achieve peak tailing factor <2, retention time between 3 to 10min and capacity factor K >3, (ICH, 2003).

Method validation study include system suitability, linearity, precision, accuracy, specificity, robustness, limit of detection, limit of quantification and stability of sample, reagents, instruments.

Table (3.2.6.1) Chromatographic condition employed as factorial design 2^3 method No1

Run	Factors			Response		
				t_R	Total plate	ASym5%
	X1	X_2	X_3			
1	40	60	1.0	9.701	13031	1.17
2	65	60	1.0	5.083	8052	1.29
3	40	65	1.0	12.716	14334	1.13
4	65	65	1.0	4.666	8864	1.5
5	40	60	1.3	7.550	9396	1.20
6	65	60	1.3	3.316	6094	1.17
7	40	65	1.3	9.866	11489	1.08
8	65	65	1.3	3.950	48621	1.33

The Chromatographic method development for the determination of Losartan potassium was optimized by applying factorial design experiment.

3.2.6.1.1 Effect of column type

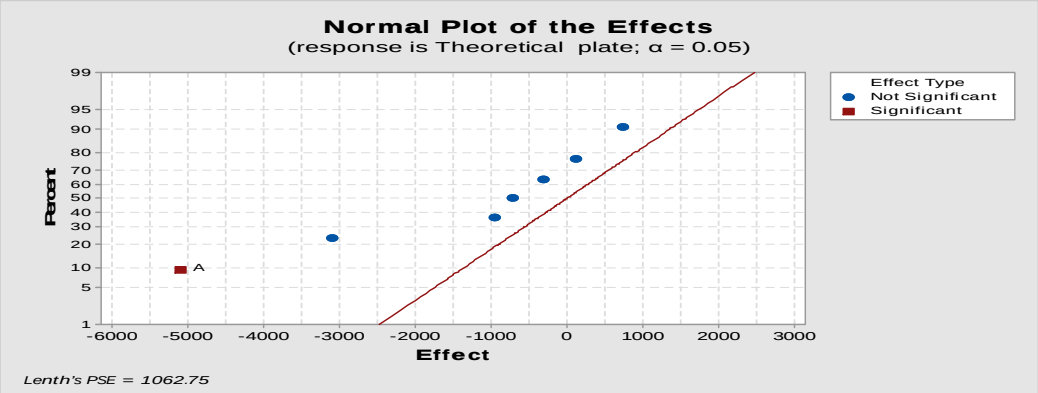
To have good separation of degradation products, we used a stationary phase. This column was found optimum hence; it become the column of work study. The best selectivity was observed on a C18 based stationary phase. The chromatographic separation was achieved using RP C18 (Kromasil 250x4.6mm100-5C1825cmx4.6mm E82860 i.d 5µm particle).

3.2.6.1.2 Effect of mobile phase Mode

Change the composition of mobile phase optimized chromatographic method by applying factorial design approach. Optimization experiments were conducted through a process of screening and optimization. The purpose of screening design is to identify the factors

that have significant effects on the selected chromatographic responses, and for this purpose a factorial design 2^3 was used to develop a stability indicating method it was necessary to maintain proportion of the mobile phase isocratic mode for determination losartan potassium peak was studied. Isocratic elution was firstly using different Acetonitrile ration with phosphate buffer pH 3.5.

By Appling statistical software (SPSS 22, Minitab 17) .The model was fit R= 90.30 if we consider P –value 0.05 and standard deviation about 1.055, acetonitrile has p-value 0.0066 is highly significant , but buffer composition no significant with respect to flow rate is mild affected the elution of peak .



Figure(3.2.6.1) resopnse of retention time at p-value 0.05

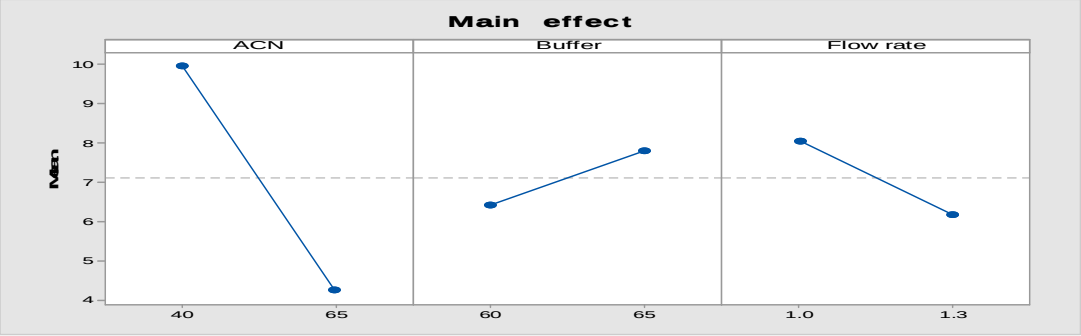


Figure (3.2.6.2) the main effect of factors at p value 0.05

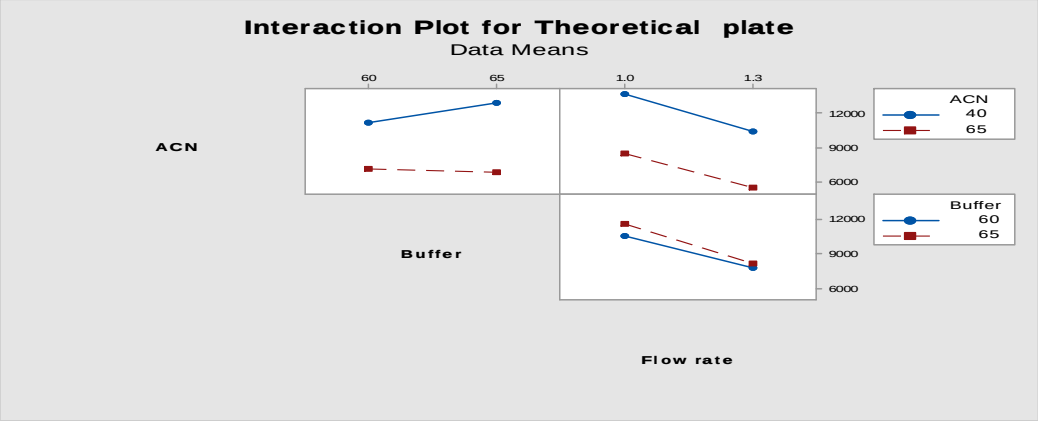


Figure (3.2.6.3) the interaction of factors at p-value 0.05

3.2.6.1.3 Effect of wavelength

The wavelength was important for detection of the components for HPLC as a detector. Losartan potassium have wavelength 250nm, this wavelength was selected as optimum wavelength.

3.2.6.1.4 Effect of Flow rate

The flow rate of mobile phase was very important to achieve the good resolution between drug and its degradation products. 1.0ml/min was efficient to resolve all peaks within appropriate time about 20 min, (Snyder *e*t *al*, and 2011).

Method validation is the process which to confirm that the analytical procedure employed for specific test is suitable for its intended use.

3.2.6.2 System suitability

System suitability parameters were measured to confirm or verify the system performance. System precision was determined on six replicate injection of standard preparation. All important characteristics were

measured, including peak resolution, capacity factor, tailing factor and theoretical plate.

3.2.6.3 Linearity and calibration Curve

The linearity of analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte.

The equation of the calibration curve of losartan potassium y =29.87x-5.772, the graph was found to be linear with correlation coefficients 0.9998, standard error of intercept 4.512 and for slope 0.558.

3.2.6.4 Precision (repeatability)

The precision of analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility .Repeatability expresses the precision under the same operating conditions over a short interval time.

3.2.6.5 Accuracy

The accuracy of analytical method is extent to which test results generated by method and true value agree .Accuracy of method was studied by recovery experiments. The accuracy of analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

The recovery was performed at three levels 80%, 100% and 120% of the label claim of losartan potassium 50 mg, placebo equivalent to one

tablet was transferred into 100ml volumetric flask and the amount of losartan potassium at 80,100 and 120% were added. Three levels were analyzed and the percentage recoveries were calculated from the calibration curve. The recovery value of losartan range from % to %, the average recovery and % RSD of three levels (nine determination) for losartan was 99.61% and 1.068 RSD.

Table (3.5.2) Results of the recovery test for the losartan potassium

3.2.6.6 Limit of detection and Quantitation (LOD and LOQ)

The detection limit of an individual analytical procedure is the lowest amount of analyte in the sample which can be detected but not necessary quantitated as exact value. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The LOD of losartan potassium was 0.176µg/ml and LOQ was 0. 534µg/ml.

Table (3.2.6.2) Calibration curve of losartan potassium

Concentration in µg/ml	Peak area at 250nm	RDS
6.4	184.18	0.96
7.2	211.24	0.52
8.0	233.11	0.42
8.8	256.26	0.82
9.6	281.15	0.94

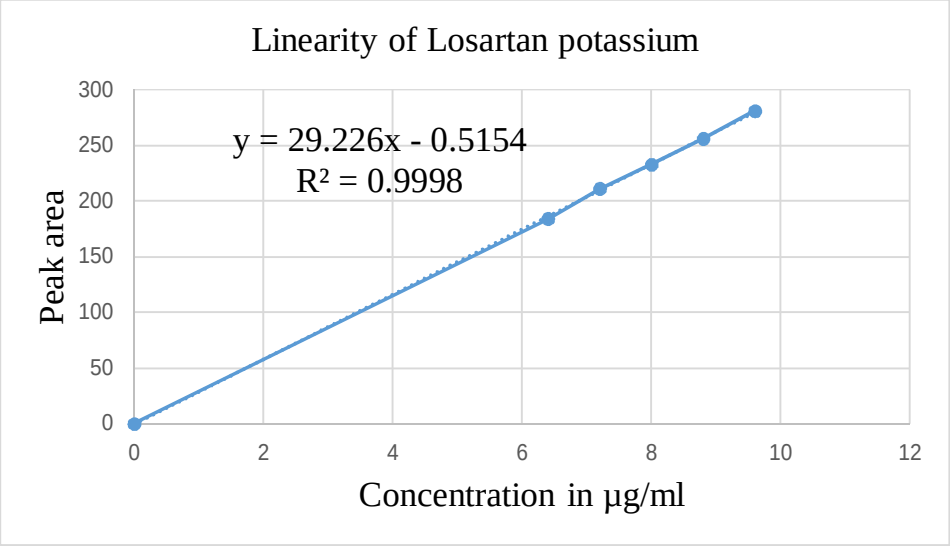


Figure (3.2.6.4) Linearity of losartan potassium

Table (3.2.6.3) Regression Characteristic and system suitability parameters of method No1

Parameter	Losartan potassium
Retention time	9.90
Asymmetry 5%	1.10
Capacity factor	4.25
Theoretical plate	6828
Linearity µg/ml	6.4 -9.6
LOD µg/ml	0.176
LOQ µg/ml	0.534
Regression equation	Y =29.87x-0.5154
Slope(m)	29.87
Intercept(c)	0.5154
Correlation coefficient (r^2)	0.9998
RSD	1.412

Table (3.2.6.4) Recovery results test for losartan potassium

Level of addition (%)	Drug	Tablet strength (n =3)µg/ml	Amount added(n= 3) µg/ml	Average Amount recovered	Recovery
80	Losartan	50	6.4	6.3	98.44
100	Losartan	50	8.0	7.99	99.87
120	Losartan	50	9.6	9.65	100.52
Average					99.61
% RSD					1.068

Table (3.2.6.5) Stress testing of Losartan potassium

S.NO	Condition	% Assay of Losartan potassium	% degradation	
			Single maximum	Total
1	No stress treatment(control sample)	99.89	Nil	Nil
2	Acid	99.80	0.50	0.10
3	Alkali	97.15	1.63	2.74
4	H_2O_2	95.45	0.57	4.53
5	UV	96.96	0.37	2.93
6	Thermal	98.77	1.87	1.12

3.2.6.7 Robustness

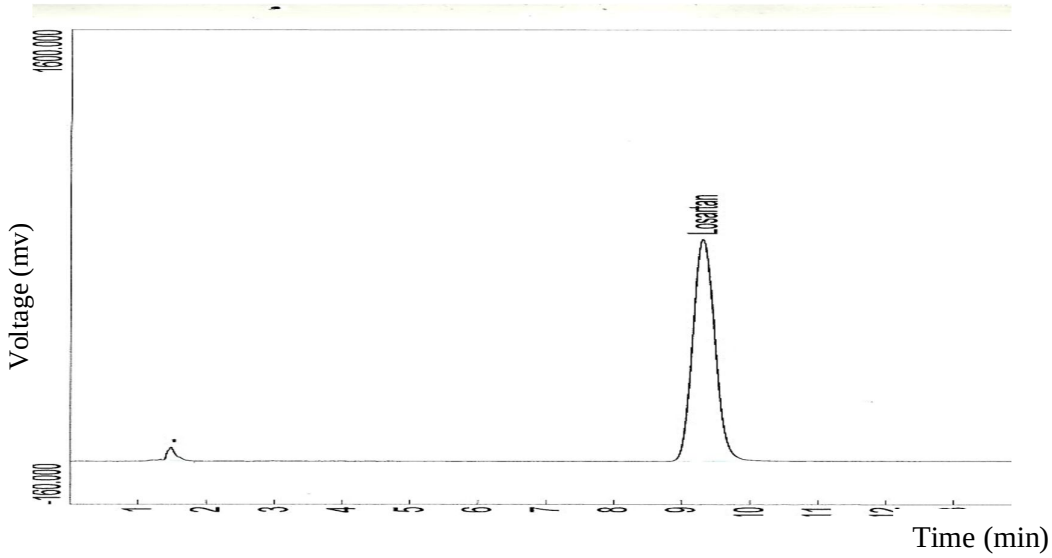
The robustness of analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. For the determination of method's robustness a number of chromatographic parameters (flow rate, buffer pH).

Table (3.2.6.6) Robustness study

Parameters	RSD of peak area
Flow rate(ml/min)	
0.9	1.175
1.0	0.778
1.1	0.485
Change the pH of buffer	
3.4	1.96
3.5	0.70
3.6	0.96

3.2.6.8 Specificity

Specificity was documented by comparing retention times obtained in the standard Losartan potassium (five samples) with those obtained from the reference Losartan potassium samples (three samples. The minimal difference between retention times (<0.1%) allows confident, highly specific, peak identification. Usually, a difference within ±3% is considered acceptable between retention times of the same amino acids present in the standard mixture and in the hydrolyzed sample, (Maria and Federico, 2006).



Figure(3.2.6.5) HPLC Chromotogram of working standard of losartan potassium

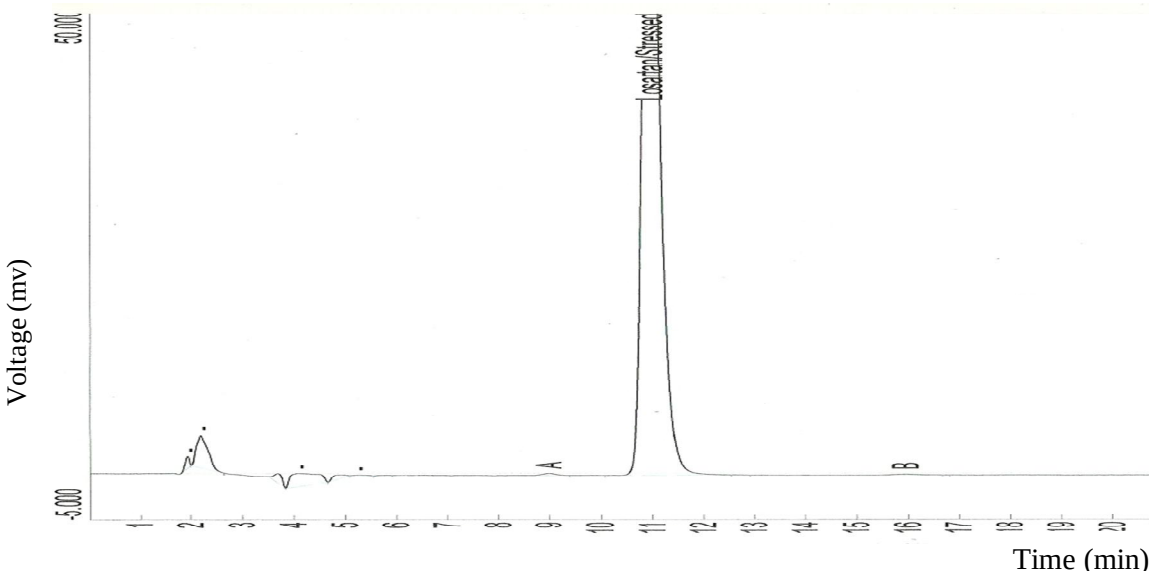


Figure (3.2.6. 6) effect of acid on instability of losartan potassium

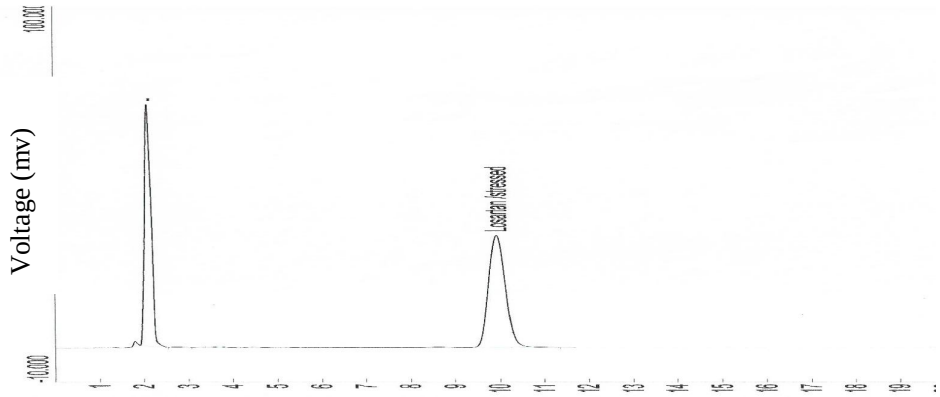


Figure (3.2.6.7) HPLC chromatogram of peroxide effect on instability of Losartan

HPLC analytical method was developed to determination of losartan potassium in pharmaceutical dosage form, the developed procedure was evaluated specificity under stress condition, linearity, accuracy, precision and robustness, the simplicity of the method allows for application in laboratories that lack sophisticated an analytical instrument such as LC-MS/MS that are complicated, costly and time consuming than a simple HPLC-UV method The method was found to be specific. Linear, precise, accurate, robust and stability –indicating. So the method is recommended for routine quality control analysis and stability sample analysis.

3.2.7 Method Validation No .2 for determination of losartan potassium

3.2.7.1 Optimization and method development

The main intention of method development is that all required chromatographic conditions are inevitably optimized.

The traditional approach to HPLC optimization is to perform an experiment by trial and error or by change one control variable at time; such method can frequently require a very large number of experiments to identify the optimal condition. Recently computer assessed to HPLC separation has addressed the problem using factorial design strategies.

In this work a three factors with two level was applied to predict the retention behavior of Losartan potassium and optimize their isocratic elution using acetonitrile as organic modifier and buffer as mobile phase.by using Mintab 17and SPSS 22 as soft ware

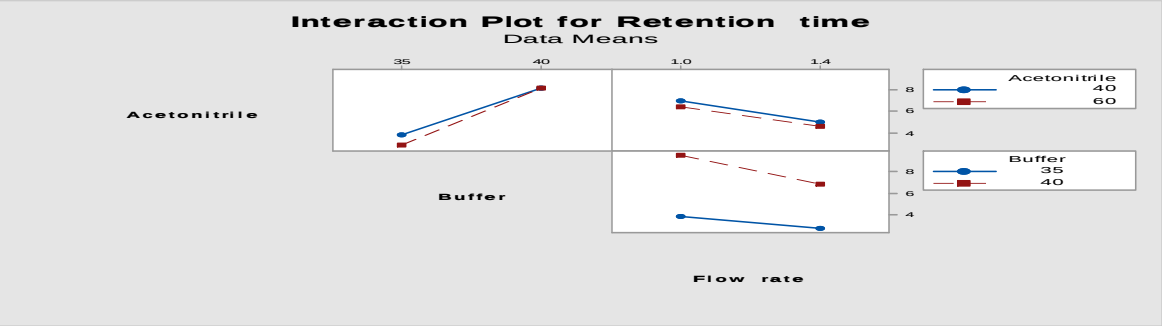
Table (3.2.7.1) Chromatographic condition employed as factorial design 2^3

Run	Factors			Response		
				Retention time	Total plate	Asym5%
	X1	X_2	X_3			
1	40	60	1.0	9.701	13031	1.17
2	65	60	1.0	5.083	8052	1.29
3	40	65	1.0	12.716	14334	1.13
4	65	65	1.0	4.666	8864	1.5
5	40	60	1.3	7.550	9396	1.20
6	65	60	1.3	3.316	6094	1.17
7	40	65	1.3	9.866	11489	1.08
8	65	65	1.3	3.950	48621	1.33

The goal of this investigation was to establishing a new simple and sensitive method that could be used in analysis degradation products of Losartan potassium.

X1, X2 and X3 factors are represent, Acetonitrile, Buffer and flow rate (-) and (+) represent the low and high of acetonitrile , buffer and flow rate.

The model summary indicate that the model was fit, R^2 =0.9961 and standard error of estimation was 0.1776 %., this for dependent variable: retention time.



Figure(3.2.7.1) interaction effect showing the influence of retention time

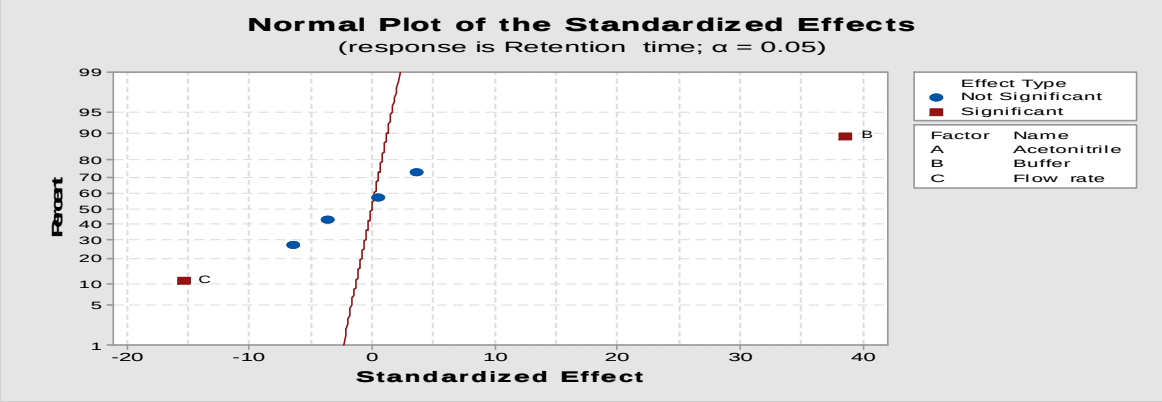
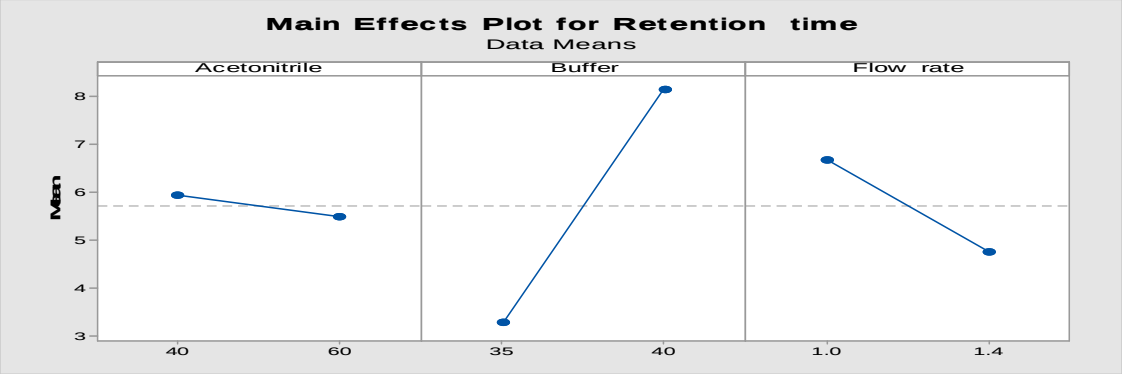


Figure (3.2.7.2) standardized effect showing the influence of retention time



Figure(3.2.7.3) main effect showing the influence of rtention time

After close observation of the concerned parameters based on the detailed results obtained and discussions of this part the following procedures were recommended for deciding losartan potassium in bulk samples as pharmaceutical formulation.

Method number 2 for determination of losartan potassium has been validated, according to guideline of ICH Q2 (R1) all parameters as discussed below were analyzed and validated accurately following the procedure of proposed method.

3.2.7.2 System suitability

During analytical method development system suitability test give an added level of confidence that the accurate mobile phase, flow rate, temperature, and column were used which ensures the system performance (pump and detector) where in parameters of system suitability such as retention time, resolution, efficiency (number of theoretical plate) and tailing factor are involved and they should be within the defined limits. The results of system suitability in respect to the proposed method are mentioned in Table (3.2.7.2).

Table (3.2.7.2) System suitability results for the proposed method

S.NO	Parameters	Results*
1	Retention time	9.98 minutes
2	Theoretical plate	3837
3	Theoretical plate per meter(t.p/m)	0.065
4	Asym 5%	1.1
5	Resolution	-

* Results for triplicate value (n =3)

3.2.7.3 Specificity

The excipients in tablets contained the following in active ingredients: Microcrystalline cellulose, lactose, maize starch and magnesium stearate as excipients, chromatograms showed that no excipients interfered with losartan potassium peak Figure (3.2.7.4), and (3.2.7.5) and (3.2.7.6).

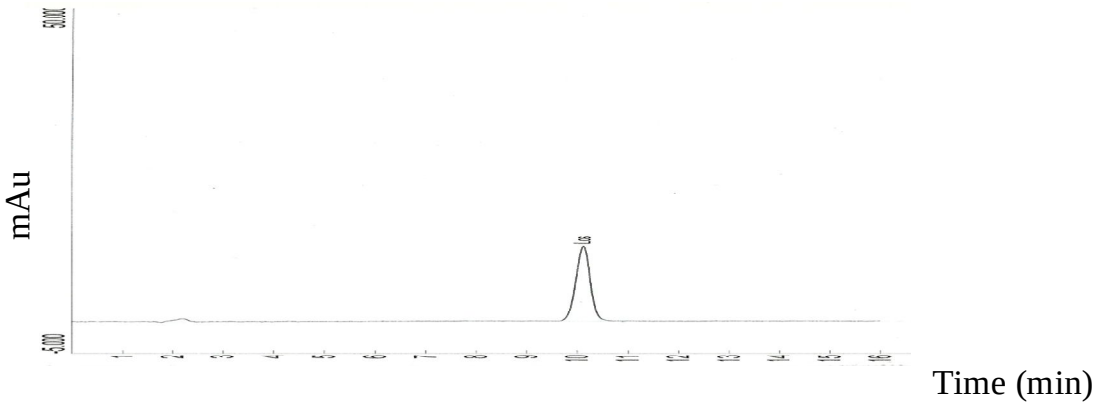


Figure (3.2.7.4) 8µg/ml HPLC chromatogram of losartan potassium tablet

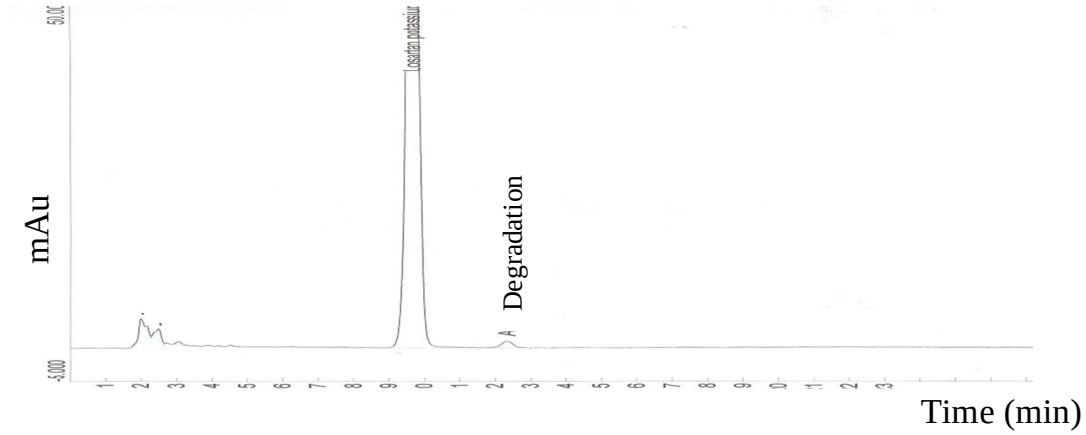


Figure (3.2.7.5) HPLC chromatogram acid degradation of losartan

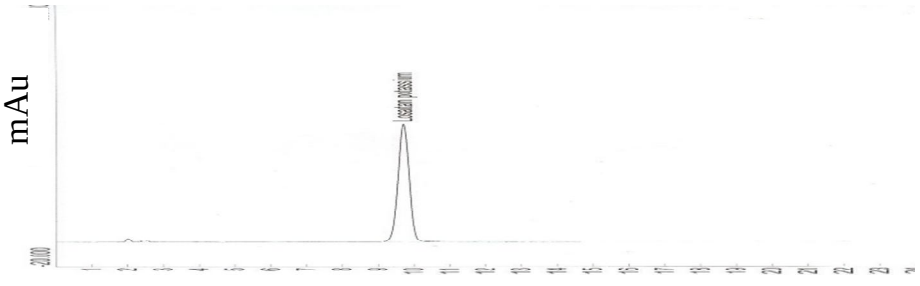


Figure (3.2.7.6) HPLC Chromatogram of Losartan potassium

3.2.7.4 Stress testing of losartan potassium

Under acidic condition losartan potassium was degradated up to 0.7 %.,under alkali stress losartan potassium was degradated up to 1.63 %,only small percent of degradation occurred when losartan exposed to UV radiation 0.37% . Oxidation and thermal degradation of losartan 0. 0.57% and 1.87% Table (3.2.7.3), which showed good indication of stability.

Table (3.2.7.3). Percentage of degradation of losartan potassium

S.NO	Condition	% Assay of Losartan potassium	% degradation
1	No stress treatment(control sample)	99.88	Nil
2	Acid	99.17	0.70
3	Alkali	98.25	1.63
4	H_2O_2	99.31	0.57
5	UV	99.84	0.37
6	Thermal	98.01	1.87

3.2.7.5 Linearity

The linearity for HPLC method was determined at five concentration levels ranging from 6.4-9.6 µ mg /mL for Losartan potassium the calibration curve was constructed by plotting response factor against respective concentration of Losartan potassium. The method of least square analysis was performed to obtain slope, intercept and correlation.

Table (3.2.7.4) linearity of losartan potassium

Level method%	Peak area at 225nm	Actual %	RSD %
80	399.6630	79.30	
80	398.956	79.16	0.28
80	401.1640	79.60	
90	463.0825	91.89	
90	459.1520	91.11	0.74
90	456.3130	90.54	
100	504.380	100.07	
100	503.350	99.88	0.10
100	504.140	100.04	
110	556.816	110.48	
110	548.022	109.00	0.68
110	552.9085	110.00	
120	600.7550	119.20	
120	597.909	119.00	0.54
120	594.933	118.00	
			Limit NMT 2%

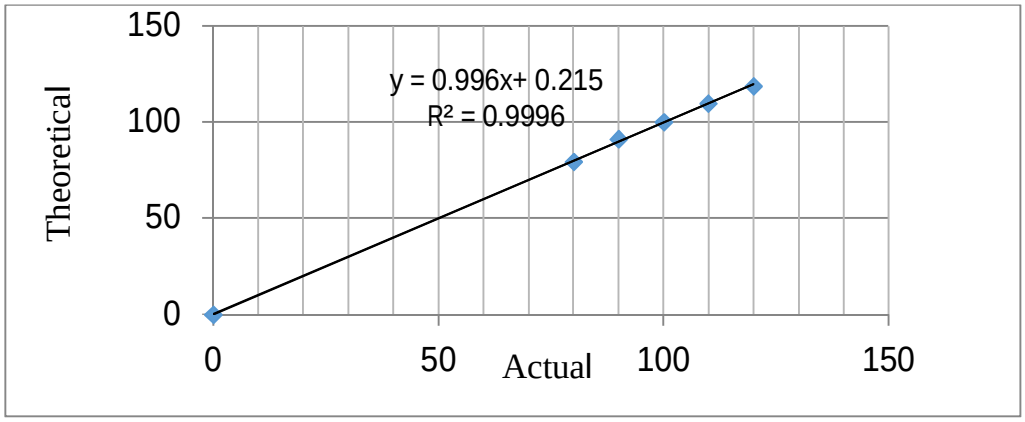


Figure (3.2.7.7) Linearity curve of losartan potassium

Table (3.2.7.5) summery of validation of losartan potassium

Parameters	Results
Linear range	6.4-9.6µg/ml
Regression equation(y=a+bx)	y = 0.996x + 0.215
Correlation coefficient(r^2)	0.9996
Slope (b)	0.996
Intercept (a)	0.215
Standard deviation of slope(s_b)	0.009
Standard deviation of intercept(s_a)	0.845
Standard error of estimation	0.934

3.2.7 .6 Precision

The main reason of study precision is to establish that promoted RP-HPLC is accurate for analyzing losartan potassium in pharmaceutical formulation as well as bulk forms.

The precision (system method) of the proposed method was evaluated by carried out of six independent assays of test sample. RSD (%) of six assay value obtained was calculated .The intermediate precision was carry out by analyzing the sample in different days.

Table (3.2.7.6) Intra-day precision result of losartan potassium

Repeatability precision		Injection no	Peak area mV.s	Assay	% RSD N=6
Sample	Concentration				
Losartan potassium	8µg/ml	1	237.66	100.22	0.44
		2	236.12	99.60	
		3	238.70	100.65	
		4	235.77	99.42	
		5	236.63	99.80	
		6	236.81	99.86	

Table (3.2.7.7) Inter-day precision result of losartan potassium

Intermediate precision		Injection no	Peak area (mV.s)	Assay	% RSD N=6
Sample	Concentration				
Losartan potassium	8µg/ml	1	238.54	100.62	0.43
		2	236.66	99.83	
		3	237.26	100.10	
		4	236.19	99.63	
		5	235.94	99.52	
		6	235.78	99.46	

3.2.7.7 Accuracy (recovery studies)

The accuracy of the HPLC method was confirmed by recovery studies by spiking 80,100 and 120% of pure drugs to the preanalysed samples and the samples after dilution injected into the system (n=3). The peak area of each drug was measured and recovery data for losartan potassium given in Table (3.2.7.8).

Table (3.2.7.8) Recovery results of losartan potassium

Amount added µg/ml	Amount found µg/ml	%Recovery	Statistical analysis of % recovery	
6.4	6.34	99.06	MEAN	99.15
6.4	6.33	99.0	SD	0.17
6.4	6.36	99.4	%RSD	0.22
8.0	8..05	100.62	MEAN	100.22
8.0	8.00	100.00	SD	0.28
8.0	8.004	100.05	%RSD	0.34
9.6	9.54	99.37	MEAN	98.96
9.6	9.52	99.17	SD	0.45
9.6	9.44	98.33	%RSD	0.55

3.2.7 .8 Robustness

Robustness of the method was determined by small deliberate changes in flow rate, and wavelength. The content of the drug was not adversely affected by these changes as evident from the low value of relative standard deviation indicating that the method was rugged and robust.

Table (3.2.7.9) robustness change of flow rate

Flow rate(ml/min)			Mean area ±SD N= 3	SEM	Mean t_R±SD	% Bias	% RSD
Original	Used	Level					
1.0	0.95	-0.05	227.27±1.81	1.28	10.12±0.097	0.0173	0.097
	1.0	0	213.57±2.06	1.45	9.794±0.0098	0.00	0.10
	1.05	+0.05	230,84±0.87	0.61	9.777±0.0098	-0.0093	0.1003

Table (3.2.7.10) robustness change of wavelength

Wavelength		SEM		%Bias	

Original	used	level	Mean area ±SD N= 3		Assay %		% RSD
225	220nm	-5	579.42±5.35	3.783	99.94	-0.022	0.923
	225nm	0	521.39±0.69	0.487	100.05	0.008	0.132
	230nm	+5	498.69±2.91	2.057	100.63	0.199	0.585

3.2.7.9 LOD and LOQ

LOD and LOQ for losartan potassium were evaluated by injecting a series of solutions and diluted with known concentrations based on the peak response and the slope of the regression equation of the parameters of LOD and LOQ were decided. The LOD of drug noticed as 0.608µg/ml and LOQ was found 1.843µg/ml .By adopting the following formula LOD=3.3(SD)/S and LOQ = 10 (SD)/S, where SD = standard deviation of response and S= slope of the calibration curve were computed.

3.2.7.10 Comparison with the reported UV spectrophotometric method:

Table (3.2.7.11) Assay results of Losartan potassium in tablets dosage form

Method	Dosage form	Declared value mg	Found value mg ±SD*	RSD	Recovery
Reference	Losacar	50	50.13±0.026	0.064	100.26
	Zyltan	50	50.07±0.024	0.061	100.14
	Cozal	50	49.99±.0.101	0.24	99.98
Proposed No.1	Losacar	50	50.15±0.104	0.25	100.3
	Zyltan	50	49.99±0.106	0.26	99.98
	Cozal	50	50.11±0.024	0.060	100.22

*Average of three determination

The results obtained above were compared with that obtained from the reported HPLC official method confirming similar accuracy and precision in the determination of losartan potassium by both methods,

Table (3.2.7.11) , for the comparison results between official method for determination of losartan potassium in dosage form and the proposed validated method number 2 we observed that, the mean of official method was 100.13 and relative standard deviation was 0.14%, with respect to validated method the mean was 100.16 and the RSD =0.16% which confirmed the fitness of the validated method.

The proposed method obeys linearity within the concentration range of 6.4 to 9.6µg/ml and coefficient correlation was found to be 0.999.The regression of the curve was Y= 0.999x+0.215.The detection and quantization limit as LOD (K=3.3) and LOQ (K=10) were calculated and the these were found to be0.608µg/ml and 1.843µg/ml respectively.

The precision measurement of intra-day and inter-day with percent relative standard deviation, 0.44% and 0.43%, which indicated that the method is highly précised.

The percentage recovery value Table (3.2.7.8), bout 99.44 % indicates the accuracy of the method .No excipients present or interference in the analysis market formulation tablets. The result of analysis of dosage form obtained were good agreement with label claim Table (3.2.7.11).

The proposed method was simple, sensitive and reliable with good precision and accuracy, the method is specific while estimating the commercial formulation without interference of excipients and other additive. Hence this method can be used for routine determination of losartan potassium in pure samples and pharmaceutical formulations.

3.3 Stability of atorvastatin calcium

3.3.1. The effect of pH- value on reaction rate of atorvastatin calcium of 80 ^{0}C

Table (3.3.1) the effect of different pH on reaction rate of Atorvastatin of 80 ^{0}C

pH - value	K at room temperature min^{-1}	K at 80 ^{0}C min^{-1}
PH2	0.03681	0.02303
PH 3	0.03681	0.02303
PH 4	0.03685	0.02533
PH 5	0.03910	0.02533
PH 6	0.03910	0.02533
PH 7	0.03910	0.02530
PH 8	0.03180	0.03220
PH 9	0.3180	0.03180
PH 10	0.03180	0.03180
PH 11	0.03180	0.03130
PH 12	0.03180	0.03130

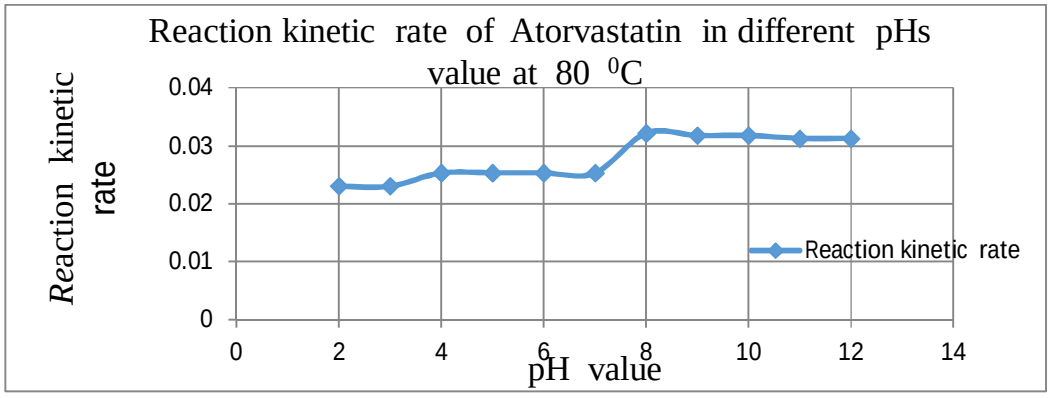


Figure (3.3.1) effect of pH on reaction rate of atorvastatin calcium of 80 ^{0}C

3.3. 2The thermal decomposition of atorvastatin calcium in acid and base medium of 80 ^{0}C

Table (3.3.2) hydrolysis of Atorvastatin Calcium in acid media method No1

Interval time / min	Remaining concentration	Log concentration
0	99.81	1.999
5	99.30	1.996
10	98.10	1.992
15	96.8	1.986
20	94.80	1.977
25	94.30	1.974
30	92.80	1.967

Table (3.3.3.) hydrolysis of Atorvastatin in alkaline media

Interval time / min	Remaining concentration	Log Concentration
0	99.88	1.99948
10	99.90	1.99957
20	99.98	1.99999
30	99.95	1.99978
40	101.0	2.0044
50	100.11	2.00047
60	100.18	2.00078

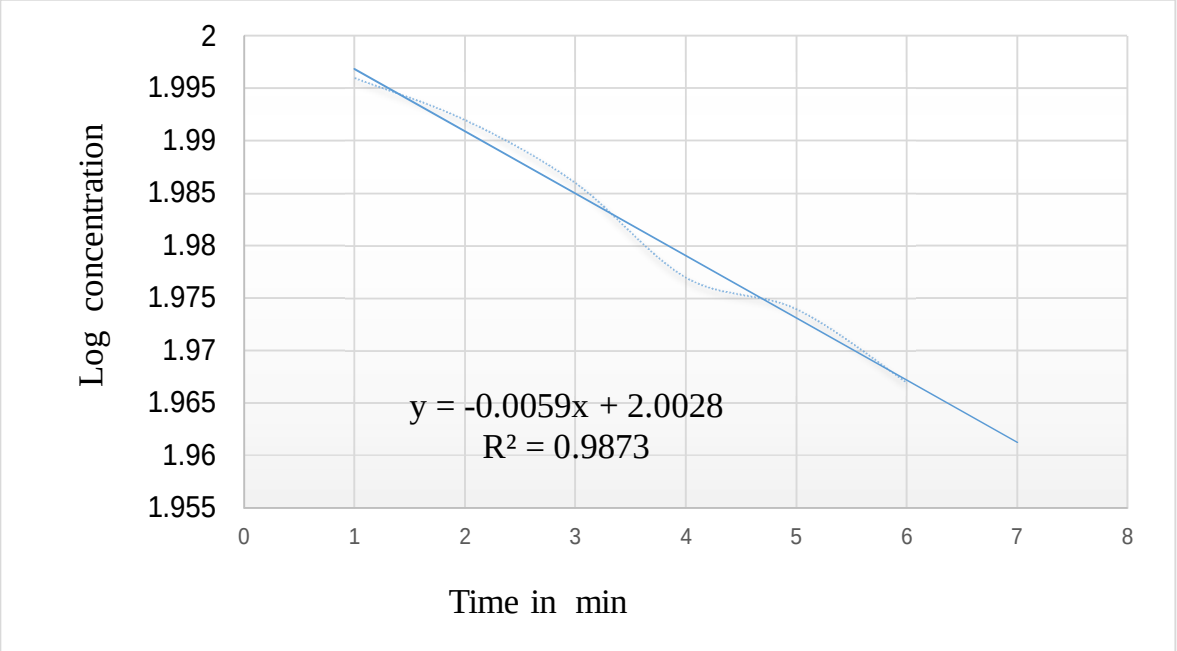


Figure (3.3.2) Hydrolysis of atorvastatin calcium in acid media

3.3.3 The effect of some excipients in reaction rate of thermal decomposition of Atorvastatin Calcium of 80 ^{0}C

Excipients are integral components of almost all pharmaceutical dosage forms. The successful formulation of a stable and effective solid dosage form depends on the careful selection of the excipients, which are added to facilitate administration, to promote the consistent release and bioavailability of the drug and protect it from degradation.

Table (3.3.4) the effect of some excipients on the reaction kinetic rate of Atorvastatin calcium of 80 ^{0}C

Excipients	Concentration	pH value	Remaining%	Kinetic reaction rate K min^{-1}
Citric Acid	0.10%	2.70	90.10	$4.606x10^{-4}$
Mangessium stearate	0.10%	4.88	98.04	$4.606x10^{-3}$
Microcrystalline cellulose	0.10%	5.45	99.71	$2.79x10^{-3}$
Lactose	0.10%	5.70	99.99	$1.381x10^{-3}$
Control	0.10%	6.66	99.93	$4.606x10^{-4}$
Tween 80	0.50%	6.80	100.00	$1.184x10^{-3}$
Acdisol	0.25%	6.85	99.17	$1.15x10^{-3}$
Calcium Carbonate	0.10%	8.40	100.50	$4.61x10^{-4}$
Disodium phosphate	0.10%	9.00	99.82	$1.3818x10^{-3}$
Mangessium hydroxide	0.10%	10.50	100.5	$1.3818x10^{-3}$

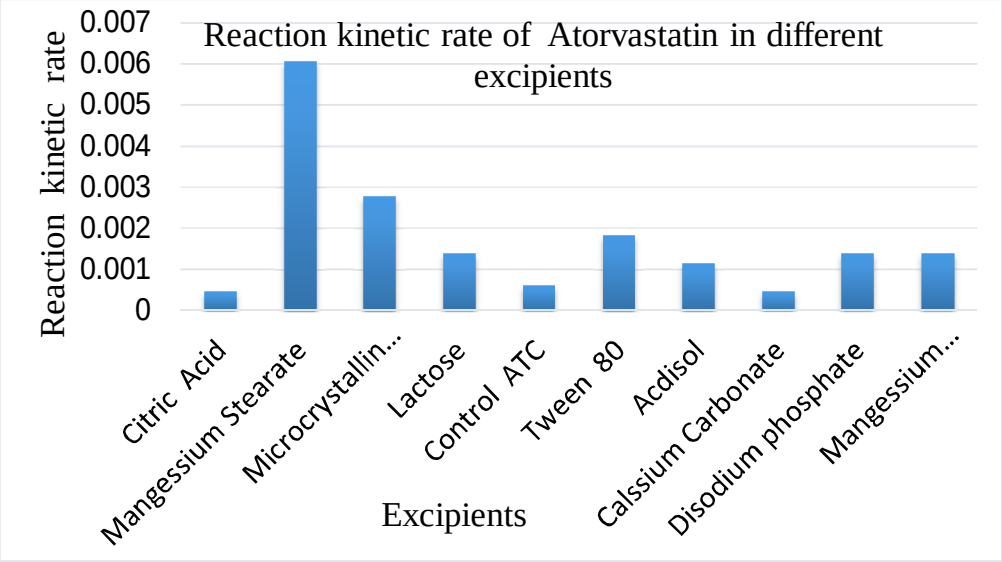


Figure (3.3.3.) Effect of excipients on reaction kinetic rate of Atorvastatin calcium

3. 3 .4 Effect of natural sunlight on instability of atorvastatin calcium in liquid state

Table (3. 3.5) Effect of natural sunlight on instability of atorvastatin in liquid state

Time in hour	Remaining concentration R.T 4.88 ±0.5min	% Deg 1 Rt 4.39±0.5min	% Deg 2 Rt 8.12 ±0.5min	Deg3 Rt 18.89±0.5min
0	99.96	-	-	-
2	96.49	4.39	8.16	18.83
4	85.40	4.43	8.08	18.93
6	74.27	4.39	8.12	18.93
8	61.27	4.40	8.22	18.90

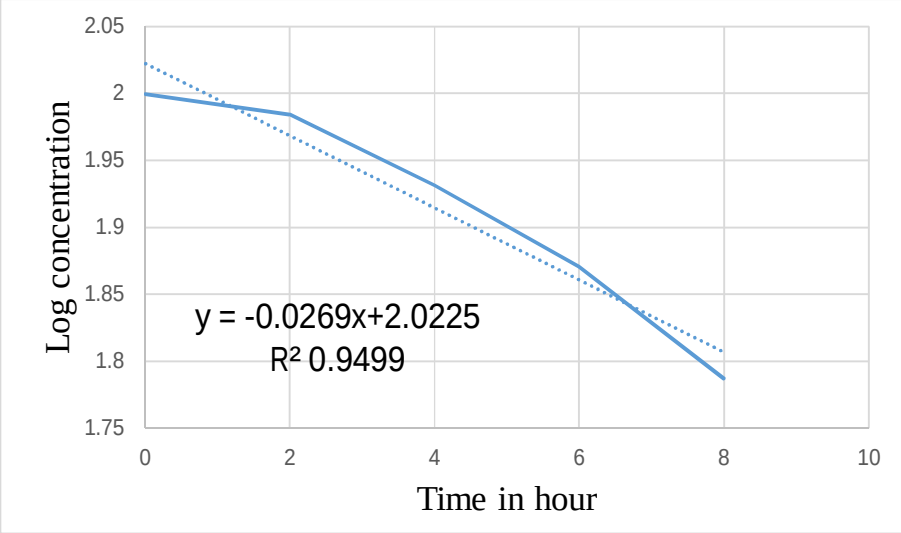


Figure (3. 3.4) Effect of natural sunlight on instability of atorvastatin (liquid state)

3.3.5 Effect of controlled temperature of 100 ^{0}C on instability of atorvastatin calcium

Table (3.3.6) The effect of temperature on instability of Atorvastatin calcium

Interval time / day	Remaining concentration	Log C	% degradation
Initial	100.07	2.00030	0.0
3	97.00	1.98677	3.07
4	95.70	1.98091	4.37

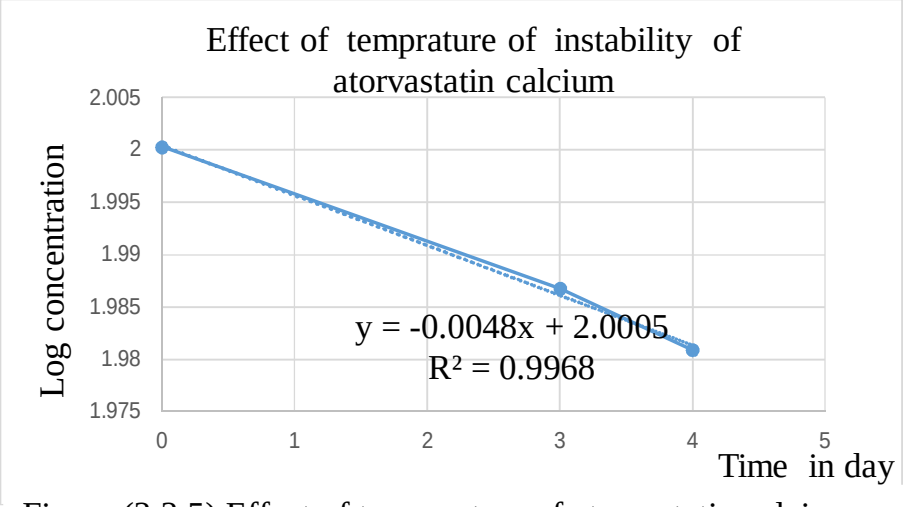


Figure (3.3.5) Effect of temperature of atorvastatin calcium

3.3.6 Water hydrolysis of atorvastatin calcium of 80 ^{0}C

Table (3.3.7) Water hydrolysis of Atorvastatin calcium method No2

Interval time / min	Remaining concentration%	Peak area	1/Concentration
Zero	99.86	1245.08	0.010014
5	100.13	1255.07	0.00998
10	100.06	1262.79	0.009994
15	99.98	1260.92	0.0100
20	100.52	1261.99	0.009948
25	101.11	1284.13	0.0099
30	99.27	1252.01	0.01007

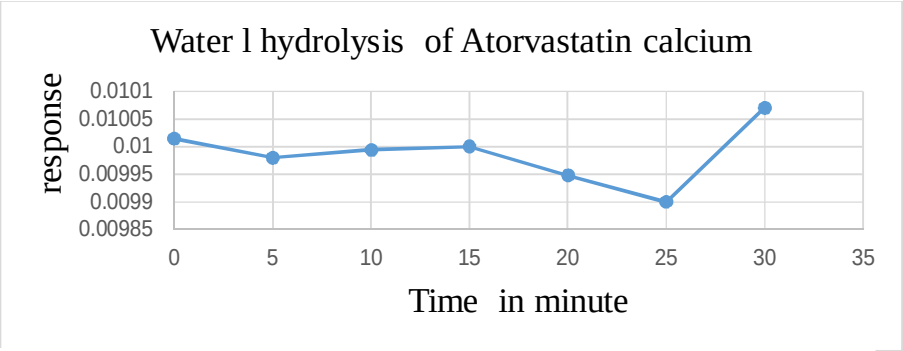


Figure (3.3.6) Water hydrolysis of Atorvastatin calcium

3.3.7 Accelerating stability study of Atorvastatin calcium of 40 ^{0}C/75% RH

Table (3.3.8) **Result** of accelerated stability testing

Condition	Drug content	% Degradation
Initial	99.88	0
One month		
Ambient	100.1	-
40 ^{0}C/75% RH	99.78	0.10
Three month		
Ambient	99.87	
40 ^{0}C/75% RH	99.58	0.30
Six month		
Ambient	100.18	
40 ^{0}C/75% RH	99.47	0.41

Stability studies and degradation kinetics are an integral parts of the quality control of a drug or medicinal product on an industrial scale. Degradation kinetics is also used to evaluate the stability under certain conditions as well as to compare stress conditions. Therefore, the intrinsic stability and kinetic studies are fundamental elements in the search for possible degradation products of drugs; however, these products do not commonly appear under normal drug storage conditions (Marcelo *et al.*, 2013).

The degradation of Atorvastatin calcium was observed in most of the stressed conditions like acidic hydrolysis, oxidative, thermal, and photolytic conditions.

Amide group in atorvastatin calcium make it unstable in acid media, the core heterocyclic pyrrole in atorvastatin calcium was susceptible to oxidation, so the quantitative NMR spectroscopy was employed for assay determination of isolation oxidation degradation products (Kracuna *et al.*, 2009).

Addition of a buffering or alkalizing like magnesium hydroxide that can provide a pH equal to or greater than (pKa +1), i.e. (pH $\geq$ 6) can enhance both solubility and dissolution rate of atorvastatin calcium different forms. (Ahjel and Lupuleasa, 2009)

Drugs as salt, their solubility is function of pH, from the chemical structure of atorvastatin find that the pKa of atorvastatin's terminal carboxyl group is (4.5). Therefore, theoretically the solubility and dissolution rate of atorvastatin calcium is markedly improved at pH values equal to or greater than (pKa +1) (i.e. pH in the gastric environment equal or greater than 5.5

Atorvastatin calcium as dosage form containing alkalizing agent (magnesium hydroxide) which is capable of increasing the pH of the gastric environment to a pH level equal to or greater than 5.5 (Klaus,2004).

Accelerating stability study of Atorvastatin of 40 ^{0}C/75% RH. According to Stability testing requirements as per ICH guidelines, accelerating stability of atorvastatin calcium, the result indicating that atorvastatin calcium was stable to this condition. Stability of Atorvastatin calcium accelerating condition showed minor degradation about 0.4% after

6 month from the initial time so atorvastatin calcium can be stable drug Table (3.3.8).

Under both acid and basic conditions, where degradation of atorvastatin calcium, considering the results in Table (3.3.2), the degradation reaction kinetics rate of atorvastatin calcium under acid conditions show first order kinetic reaction and zero order kinetic under basic condition, Table (3.3.3), in the acid medium, the first order represented the kinetics of best fit, which presented a linear correlation coefficient (r) of close to 0.9873. According to this model, the rate constant (k) for atorvastatin calcium was 0.01358 min−1. According to the results, it could be observed that the atorvastatin calcium undergoes hydrolytic degradation.

Atorvastatin calcium was found to be stable under accelerating condition Table (3.3.9) for 6 months, no significant change in assay value with consider the initial value 99.88% and final assay 99.47%.

Drugs with functional groups such as esters, amides, lactones or lactams may be susceptible to hydrolytic degradation. It is probably the most commonly encountered mode of drug degradation because of the prevalence of such groups in medicinal agents and the ubiquitous nature of water. Water can also act as a vehicle for interactions or facilitate microbial growth.

Photochemical reaction decompose involves free radical and ion and hence will strongly affect the molecules of the drug. Atorvastatin calcium was very sensitive to UV radiation at wavelength 254 nm when exposure for 16 hour in liquid state, in this reaction the molecule absorb light (photon) at 254nm, which cause increasing energy causes decomposition.

Natural sunlight lies in wavelength range (290-780nm) of which only higher energy UV range (290-320nm) cause photo degradation (Godse *et al.*, 2009).

Photolysis is the process by which light-sensitive drugs or excipient molecules are chemically degraded by light, room light or sunlight. Ultra violet light causes more damage than red/orange light. The variation of degradation depends on the wavelength of light, shorter wavelengths because more damage than longer wavelengths. Before a photolytic reaction can occur, the energy from light radiation must be absorbed by the molecules. A photosensitizer is the drug or excipient molecule that absorbs light energy. Two way in which photolysis can occur are: the light energy absorbed must be sufficient to achieve the activation energy or the light energy absorbed by molecules is passed on to other molecules Drug degradation- where the breakdown of a drug is a chemical reaction involving the collision of molecules (Gilbert *et al.*,2002).

Atorvastatin calcium more decomposed when exposure to direct sunlight for 7 hours Table (3.3.5), Figure (3.3.4) the reaction rate about 0.062 $min^{-.}$ Atorvastatin calcium undergoes degradation at controlled temperature of 100 ^{0}C atorvastatin showed reasonable degradation after kept at oven for 4 days the 4.37 % with respect to the initial concentration 100.07%, Table (3.3.6). In neutral or water hydrolysis atorvastatin calcium has no appreciable degradation observed Table (3.3.8).

At controlled temperature 100 ^{0}C atorvastatin calcium reasonable degradation observed 4.37% compared to the initial percentage at zero time Table (3.4.6).

Atorvastatin calcium was more stable in alkaline media of temperature 80^0C due to highly electron density in presence of amide group and benzene ring, so the nucleophile cannot attack the core of the molecule. The reaction kinetic rate about 0.01924 min^{-1} compared to hydrolysis in acid media, the reaction kinetic rate about 0.01358 min^{-1} Table (3.3.2).

Atorvastatin calcium is susceptible to oxidation, sometimes active drug may retain its potency in presence of excipients and some excipient may lead to compromising the integrity of drug product, citric acid as antioxidant incorporated to the formula to suppress the oxidation so citric acid will make the active ingredient more stabilization.

Since atorvastatin calcium is susceptible to oxidation, antioxidants can be incorporated for its stabilization, citric acid in concentration 0.1%, pH 2.70 degradated the atorvastatin calcium 9.83% with reaction kinetic rate 4.606 $x10^{-3}$ and magnesium stearate pH 4.88, fastening the reaction, corresponding in degradation 1.89% compared to the control sample kinetic reaction rate 4.606 $x10^{-4}$ min. Alkalizing agents as used in the present invention increase the pH of the formulation, when such formulations are added to water, magnesium hydroxide as excipient has pH 10.5 in concentration 0.1% more stable and the reaction kinetic rate about $1.3818x10^{-3}$.The remaining concentration 100.5 % with respect to the control 99.93%., Table (3.3.3) and Figure(3.3.4). From the above date decreasing pH value increasing the degradation rate.

When considering drug stability it is important to consider the individual functional groups present in the drug molecule and the environment they find themselves.

A drug may be stable once in the blood stream but be unstable in the highly acidic environment of stomach. Understanding the reactivity of individual functional groups provides a valuable insight into how a drug molecule congaing multiple functional group will behave (David, 2014). Certain functional groups in drug molecules are prone to chemical degradation, drug with an ester functional group are easily hydrolysis and many drug molecules are susceptible to oxidation (atorvastatin calcium) which have certain oxidizable -OH group. Amide in atorvastatin calcium make it unstable in acidic media. The effect of solvents on solubility of atorvastatin calcium.

The water hydrolysis of atorvastatin calcium of 80 ^{0}C at thermostatic water bath atorvastatin calcium showed no degradation after 30 minute, 99.27% compared to the initial percentage 99.86%.

Atorvastatin calcium in buffering system pH 2 to PH 12 its reaction rate increased from pH 5 to pH8 at 80 ^{0}C controlled temperature, kinetic reaction rate (0.02533, 0.0322 min^{-1}) respectively Table (3.3.1) and Figure (3.3.1).

3.4 Stability of losartan potassium:

Safety and efficacy of pharmaceuticals are two fundamental issues of importance in drug therapy. Instability of pharmaceuticals can cause a change in physical, chemical, pharmacological and toxicological properties of the active pharmaceutical ingredients (API), thereby affecting its safety and efficacy. Hence, the pharmacists should take cognizance of various factors such as drug stability, possible degradation products, mechanisms and routes of degradation and potential interactions with excipients utilized in the formulation to ensure the delivery of their

therapeutic values to patients. In order to assess the stability of a drug product, one needs an appropriate analytical methodology, so called the stability indicating methods which allow accurate and precise quantitation of the drug, its degradation products and interaction products, if any. Forced degradation studies were performed on Losartan potassium to prove the stability indicating property of the method (Janardhanan *et al.*, 2011).

Information on the stability of the drug substance is an integral part of the systematic approach to stability evaluation. Stress testing (or forced degradation studies) is a critical component of the drug development process .The ICH guideline indicates that stress testing is designed to help "determine the intrinsic stability of the molecule by establishing degradation pathways in order to identify the likely degradation products and to validate the stability indicating power of the analytical procedures used . Stress testing also is becoming increasingly important in testing new molecules. Methods developed by stress testing and the stability information gained from those methods can have a significant effect on the actual compound selected for development .Literature review revealed that several methods have been reported for analysis of the drug in pharmaceutical formulation alone or with other drugs in combination. USP described a HPLC method for assay and impurities of Losartan potassium, (USP, 2011), (BP, 2014).

3.4.1 The effect of temperature on reaction kinetic rate of losartan potassium at different pH – value:

Table (3.4.1) the effect of pH value on reaction kinetic rate of losartan

pH - value	K at room temperature	K at 80 ^{0}C min^{-1}
PH2	1.1515x10^{-4}min^{-1}	2.727x10^{-3}min^{-1}
PH 3	2.303x10^{-4}	1.1515x10^{-3}
PH 4	2.341x10^{-4}	2.727 x10^{-4}
PH 5	2.303x10^{-4}	4.606x10^{-4}
PH 6	2.303x10^{-4}	1.1515x10^{-3}
PH 7	4.606x10^{-4}	6.909x10^{-4}
PH 8	2.303x10^{-4}	4.606x10^{-4}
PH 9	2.303x10^{-4}	2.303x10^{-4}
PH 10	1.381.x10^{-4}	4.606x10^{-4}
PH 11	1.8424x10^{-4}	2.303x10^{-4}
PH 12	1.909x10^{-4}	2.303x10^{-4}

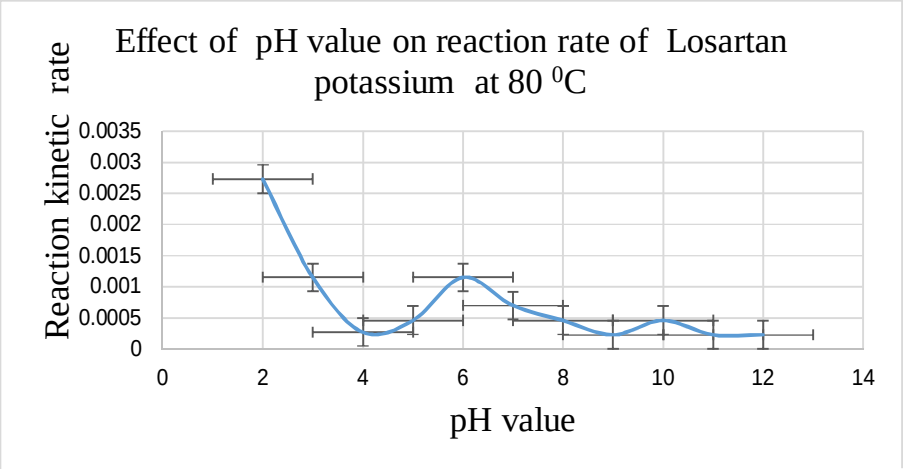


Figure (3.4.1) effect of pH on instability of Losartan potassium at 80 C^0

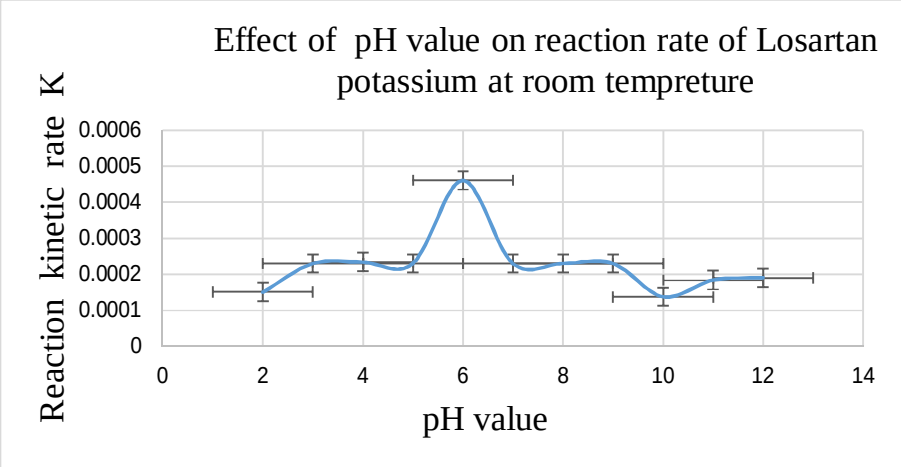


Figure (3.4.2) effect of pH on instability of Losartan at ambient temperature

3.4.2 The effect of some excipients on reaction rate of losartan potassium:

Most excipients have no direct pharmacological action but they can impart useful properties to the formulation. Physical and chemical interactions between drugs and excipients can affect the chemical nature, the stability and bioavailability of drug products, and consequently, their therapeutic efficacy and safety , acid drugs become more non ionized in acidic pH environment, so far focus on the structure of losartan potassium , it possess soluble functional group so its soluble in water, ionized molecule. On easily released relatively at this pH, above neutral pH losartan potassium decomposition rate increase, (Rowe, 2009).

Table (3.4.2) effect of excipients kinetic reaction on losartan potassium of 80 ^{0}C

Excipient	Concentration%	pH value	Remaining %	Kinetic reaction rate K^{-1}
Povidone K 30	0.1%	3.5	100.80	$4.606x10^{-4}$
Mangessium stearate	0.1%	4.8	100.03	$1.16x10^{-3}$
Microcrystalline cellulose	0.1%	5.45	100.15	$2.99x10^{-3}$
Lactose	0.1%	5.7	98.96	$4.61x10^{-4}$
Maze starch	0.1%	5.8	97.00	$1.152x10^{-3}$
Talc powder	0.1%	6.5	97.75	$2.76x10^{-3}$
Control	0.1%	7.4	95.00	$1.15x10^{-3}$

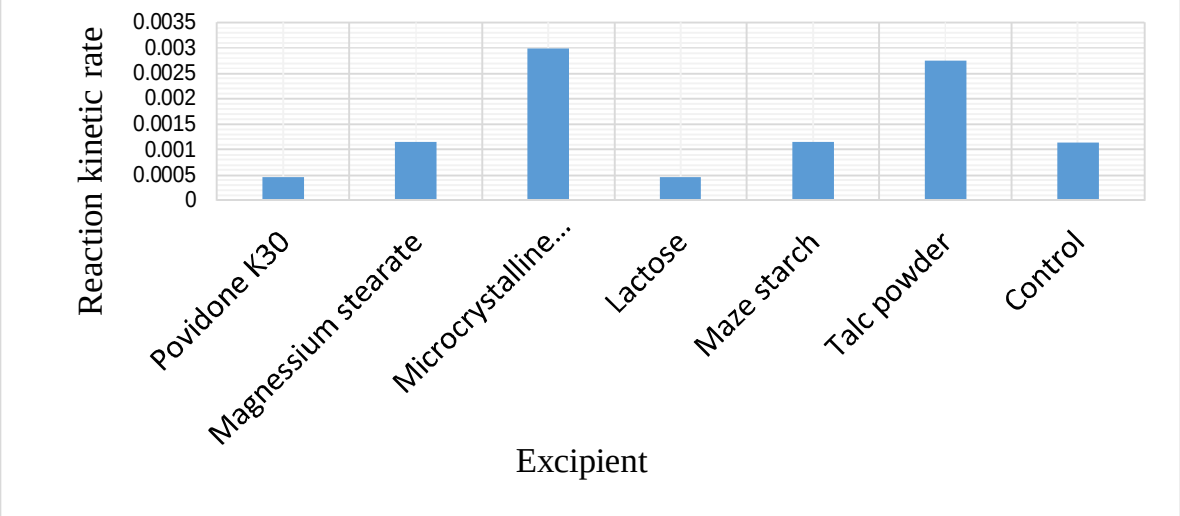


Figure (3.4.3) effect of some excipients on reaction rate of losartan potassium of 80 ^{0}C

3.4.3 Thermal effect of acid or base on instability of losartan method No1

Table (3.4.3) effect of acid on instability of losartan method No1

Interval time / min	Remaining concentration
0	100.35
5	100.12
10	99.87
15	100.1
20	100.05
25	99.95
30	99.86

Table (3.4.4) effect of base on instability of losartan

Interval time / min	Remaining concentration	Log Concentration
0	100.35	2.0015
10	99.61	1.9980
20	98.54	1.9940
30	98.25	1.9921
40	97.58	1.9893
50	97.00	1.9867
60	96.5	1.984

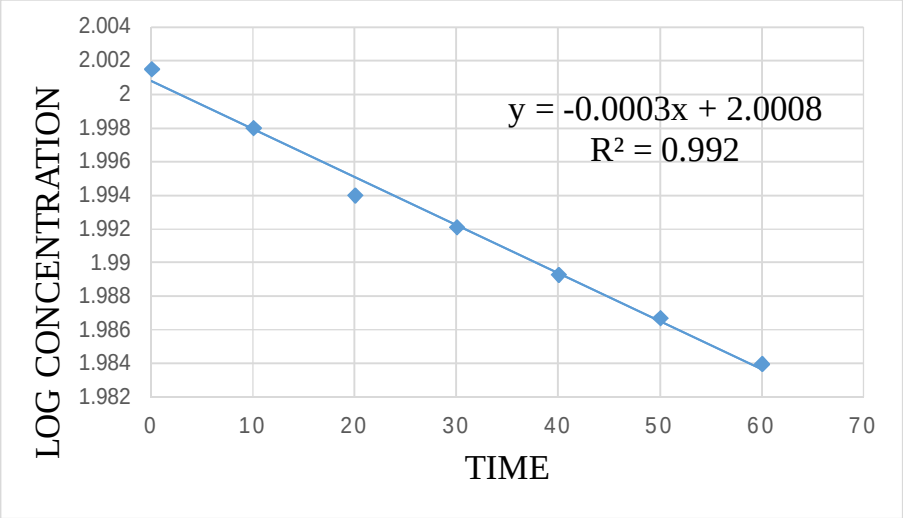


Figure (3.4.4) effect of base on instability of Losartan potassium

3.4.4 Accelerated stability studies of losartan potassium:

Table (3.4.5) Accelerating stability of losartan potassium

Condition	Drug content	% Degradation
Initial	99.89	0
One month		
Ambient	99.9	-
40 ^{0}C/75% RH	99.00	0.89
Three month		
Ambient	100.2	
40 ^{0}C/75% RH	96.45	3.44
Six month		
Ambient	100.1	
40 ^{0}C/75% RH	86.40	13.49

3.4.5 Effect of natural daylight on stability of losartan potassium (liquid form):

Table (3.4.6) the effect of natural daylight of losartan potassium (liquid state) analyzed by method No2.

Time in hour	%Remaining	Log C
0	100.22	2.0009
1	100.16	2.0007
2	100.12	2.0005
3	100.10	2.0004
4	100.14	2.00061
5	100.13	2.0005
6	100.15	2.0006
7	100.11	2.0004
8	100.18	2.0008

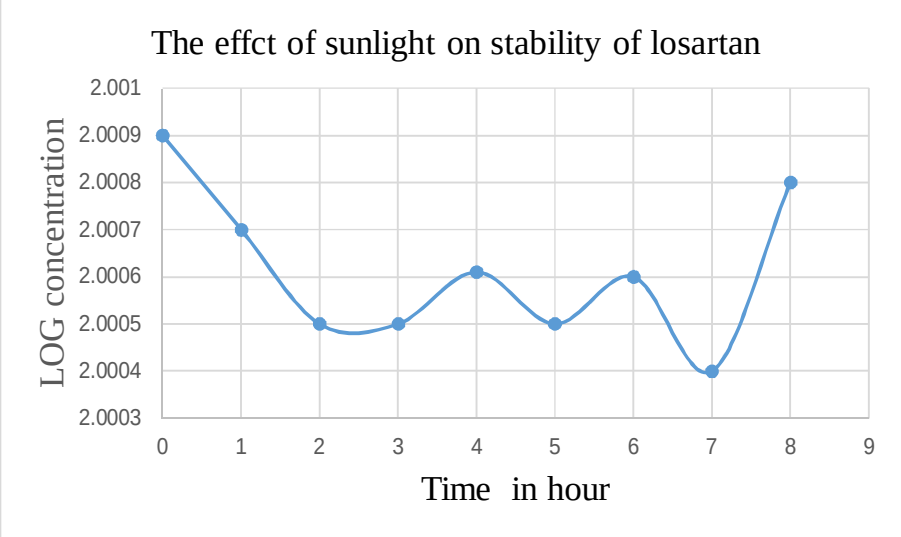


Figure (3.4.5) effect natural daylight of losartan potassium in liquid state.

3.4.6 Thermal decomposition of losartan potassium in acid or base:

Table (3.4.7) Thermal decomposition of losartan potassium reflexed in acid or base, method No 2.

Condition	Drug content	% degradation
Initial	100.14	0.0
Reflexed in acid	99.77	0.37
Reflexed in base	97.25	2.89

3.4.7 The effect of UV radiation on instability of losartan potassium in liquid form. , method No1

Table (3.4.8) the effect of UV radiation on stability of Losartan potassium method No.1

Time in day	Concentration	1/C	Degradation%
Zero	99.81	0.010020	0.0
1day	95.4	0.0104082	4.41
2days	94.8	0.010548	5.51
3days	94.60	0.0105708	5.52
6 days	94.48	0.010584	5.33

3.4.8 Comparison of analytical results between official BP method and validated methods No.1 and No.2

Table (3.4.9) Comparison of analysis result of UV radiation effect on losartan between official BP and validated methods No .1 and No.2

Time in day	Official method			
	Conc.%	t_R	Asym 5%	Total plate
Initial	99.56	7.10	1.31	3422
Day 1	94.89	7.05	1.54	3506
Day 2	94.62	7.30	1.54	2776
Day 3	94.2	7.20	1.38	2839
Day 6	93.6	7.20	1.39	2762
	Validated method No. 1			
Initial	100.14	9.98	1.06	7200
Day 1	96.35	10.05	1.12	6176
Day 2	95.15	10.00	1.12	6422
Day 3	93.60	9.97	1.1	6584
Day 6	92.88	9.95	1.12	6864
	Validated method No. 2			
Initial	99.81	9.96	1.10	8272
Day 1	95.40	9.98	1.13	8512
Day 2	94.80	10.10	1.10	8309
Day 3	94.60	9.98	1.11	8087
Day 6	94.48	10.00	1.10	8055

The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors, such as temperature, humidity, and light, and to establish a retest period for the drug substance or a shelf life for the drug product and recommended storage conditions.

The stability studies indicates that appreciable changes were observed by treating the drug with UV light, Thermal stress, oxidation and basic hydrolysis, however there was no appreciable change with acidic degradation. The proposed methods are satisfactory to determine the degradation of losartan under UV radiation table (3.4.9), comparing to the official BP method regarding to the asymmetrical of peaks, total plate and retention time which expresses good reproducibility with RSD 0.38 for method No.1 and 0.55% for method No.2.

Forced degradation study was performed to ensure that the degradation products generated due to stress condition are not interfering with the API peak. API and drug product should be forcefully stressed by treating with acid, alkali, hydrolysis, photolysis and oxidizing agent by controlling stress agent concentration and exposure time to achieve degradation about 10-30%. Achieving 100% degradation was avoided because it would be too strenuous and possibly cause secondary degradation giving degradated of the API and or excipients. The possibility of such stress may not go to happen under normal storage conditions, (Kim, 2008).

According to the structure of losartan potassium figure (1.2), imidazole is heterocyclic compound is the core of losartan potassium, two phenyl group attached to tetrazole group which has pKa (4.29). Losartan potassium has pharmacophore group like imidazole ring, biphenyl group and tetrazole moiety, tetrazole is highly stable to heat. Biphenyl group is neutral molecule (due to absence of active functional group moiety on it , it's fairly non-reactive due to lack of functional group and stable compound to decompose biphenyl insoluble in water. Imidazole is polar, miscible with water and other solvents, basic in nature (Chen *et al*., 2014).

The Accelerated stability studies for losartan potassium Table (3.4.5), confirmed that t losartan potassium was susceptible to moisture and heat.

Light can change the properties of biomolecules and the number of drugs found to be photo chemically unstable is steadily increasing. The effects of light on drugs include not only degradation reactions but also other processes, such as the formation of radicals, energy transfer, and luminescence. Adequate protection for most drug products during storage and distribution is needed. Indeed, proper storage conditions that secure protection from UV and visible radiation are essential for the efficacy of many common dermatologic drugs. If a drug is exposed to fluorescent tubes and/or filtered daylight for several weeks or months before it is finally administered to the patient, the drug may be altered. The most common consequence of drug photodecomposition is loss of potency with concomitant loss of therapeutic activity. It is therefore of interest to be aware of light induced reaction in biomolecules.

Losartan potassium in liquid form directly degradated when the time of exposure increased table (3.4.8), so losartan potassium was sensitive to UV radiation, degradate results from destruction of the imidazole ring of losartan potassium. (Maria *et al.,* 2012).

The stability of losartan potassium when exposed to direct sunlight for 8 hours it showed no degradation in liquid state which was assured that losartan potassium is thermal stable drug Table (3.4.6) and Figure (3.4.5).

Thermal treatment of losartan potassium reflexed in acid or base confirmed that losartan was stable in acid media degradation 0.37% and less stable toward base media 2.89 % .degradation Table (3.4.5).

Degradation was not observed when Losartan potassium was reflexed in base or acid media, no major degradation observed, due to steric hindrance of losartan potassium from tetrazole group and butyl group, we can say that losartan was stable drug.

Buffering agent can be necessary to maintain pH of the formulation in order to ensure physiological compatibility and maintaining chemical stability and optimizing solubility of drug, pH influence the rate of decomposition of most drugs, drugs are stable between pH4 and 8. Weakly acidic and basic drugs shows good solubility when they are ionized and they also decomposed faster when they are ionized (Rina and Kinjal, 2012).Variation in pH value from pH 4 to pH 9 in aqueous losartan solution did not alter the reaction kinetic rate at room temperature Figure (3.4.2), while shown in Figure (3.4.3) the reaction rate increased at pH2, 3 and 6 whereas it has decreased over pH 8 to 12 at temperature of 80 ^{0}C Table (3.4.1).

Excipients are pharmacologically inactive substances included in tablet dosage form, which is used as carrier of active ingredient. An excipient is a natural or synthetic substance formulated alongside the active ingredient of a medication, included for the purpose of bulking-up formulations that contain potent active ingredients. Microcrystalline cellulose its pH 5.45, increased the reaction kinetic rate of losartan potassium at 80 ^{0}C but the remaining of drug concentration no degradation on the contrary talc powder its pH 6.5 increased the reaction rate of losartan potassium but the drug active ingredient has degradated Figure (3.4.3) and Table(3.4.2).

As its thump the effect of pH of excipient of the stability of losartan Table (3.42) denote that, when the pH of excipient decrease the stability of

losartan increased vice versa , when the pH of excipient increase , the stability of losartan decreased Figure (3.4.3.) regarding to the control sample without excipient so that losartan pH dependent.

Losartan potassium show stability in acid media when reflexed 0.37% degradation which compered to, the reflexed in base media 2.89% Table (3.4.7)

3.5 Conclusion and recommendations

Column chemistry, solvent selectivity (solvent type), solvent strength in the mobile phase, additive strength, detection wavelength, and flow rate were varied to determine the chromatographic conditions giving the best separation. A new stability indicating methods was developed and validated for determination of atorvastatin calcium and losartan potassium in pharmaceutical dosage form in the presence of degradation products, the column used was C_{18} (250x4.6 mm, 5µmm).

The chromatographic separation was achieved on isocratic elution mode, the detection wavelength was 246nm for atorvastatin and 250,225 nm for losartan potassium.

The total run time for atorvastatin about 5.0min and losartan 10.0min respectively.

The methods developed and optimized applying factorial design approach , which enable highly efficiency for proposed methods in short way, further more save time and minimizing effort and reduce the consumption of reagents. The validation RP- HPLC methods are validated for precision. Accuracy, linearity, robustness and found to be precise, accurate, linear, robust and stability –indicating methods., the 2^3 Full factorial design was applied to screen the effect of the mobile phase

composition a separation efficiency, retention time, peak height, peak area and base line resolution.

After optimization and validated the HPLC methods, the mobile phase was immiscible in stationary phase this case is practically important as its description popular process interaction between mobile phase and stationary phase of the most of the process, when the distribution factor K K= C_s/C_m) was constant, does not change with solute concentration, we have Gaussian-type peak and retention times independent of amount of analyte injected in this circumstances we have linear chromatography.

Methanol and acetonitrile are popular solvents used in the reverse phase liquid chromatography, in this study, primary methanol and phosphate buffer offered bad peak tailing of analyzing atorvastatin and losartan potassium individually, but acetonitrile with different concentration separated the good peak with highly total theoretical plate.

HPLC method found to be suitable in analytical of degradation of atorvastatin calcium but not enough to analyze the decomposition of losartan potassium

Thus the proposed methods can be employed for assessing the stability of atorvastatin calcium or losartan potassium for its individual dosage form or combination.

UV spectrum of atorvastatin calcium and losartan potassium in different solvent s depended on the polarity and strength of solvents used, i.e. polarity. First derivate technique was to be suitable for estimation atorvastatin calcium and losartan potassium in bulk and pharmaceutical dosage form.

Atorvastatin calcium found to be readily degradated in acid medium ; on the other hand stable towards basic medium, thermally stable when the active ingredient subjected to heating over 80 ^{0}C.

Thermally stability of atorvastatin calcium in solid form at 100 ^{0}C for 4 days was found to be degradated about 4.37% Table (3.4.1).

Accelerating stability studies of atorvastatin indicated that the percentage of the active ingredient was stable during 6 months according to the ICH guideline, 40ºC ±2ºC/75% ±5%RH, which proved that atorvastatin calcium was stable, but showed minor degradation about 0.41% from the initial content at zero time 99.88 Table (3.4.1).

Atorvastatin calcium was insoluble in water so its solubility was pH dependent.

Atorvastatin calcium thermally decomposed in acid medium showed first –order reaction kinetic rate and zero order in basic medium

Some excipients affected the degradation of atorvastatin calcium thermally in acid condition like citric acid, which degradated the active ingredient of atorvastatin calcium about 10 %, therefore atorvastatin calcium formulated in pH >than 6.0.

Atorvastatin calcium in liquid state affected by natural sunlight when exposed for 8 hours Table (3.4.1).

Due to physiochemical properties of Losartan potassium it seemed to be more stable drug toward stress condition except when conducted at accelerating condition 40ºC ±2ºC/75% ±5%RH ;Table (3.4.1) losartan found to be unstable toward heat and humidity .

Losartan potassium thermally decomposed when increased the pH of excipients increased with respect to the active ingredient as control sample Table (3.4.2).The official BP method compared to the proposed HPLC method was satisfactory, system suitability was achieved with both

methods but the theoretical plate >2000 and good peak asymmetry when compared to official method Table (3.4.1).

UV spectrophotometric derivatisation was used accurately precise to determination of atorvastatin calcium and losartan potassium in tablet dosage form in routine work.

Analysis of losartan potassium degradation products needed sophisticated instruments like LC-MS/MS to investigate the degradations.

The degradation of losartan potassium was not achieved with isocratic method by using DAD, atmospheric pressure chemical ionization APCI in the LC MS/MS analysis could be used (Zhao *et al*., 1999).

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