Analytical Method Development and Validation of a Combination Formulation

A project submitted

by

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to

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Dedicated to my parents

Certification Statement

This is to certify that this project titled 'Analytical Method Development and Validation of a Combination Formulation' submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Dr. Eva Rahman Kabir, Associate Professor, Department of Pharmacy, BRAC University and that appropriate credit is given where I have used the language, ideas or writings of another.

Signed,

Noshin 20.03.15.

Countersigned by the supervisor

E Rahman 20/3/15

Acknowledgement

The blessings and mercy of the Almighty who is the source of our life and strength of our knowledge and wisdom, has helped me to continue my study in full diligence which I hope will reflect in my project.

This research could not also have been completed without the support of many people who are gratefully acknowledged here.

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Abstract

Hypertension and dyslipidemia may frequently coexist, and together have an increase in coronary heart disease related events. Combination therapy of rosuvastatin calcium and amlodipine besylate, effective for the control of hypertension by substantially reducing blood pressure and cholesterol levels, can improve its control rates to well above 80% rather than a single pill for hypertension which will control no more than 50% of a hypertensive population. The objective of the present study was to develop and validate a simple, selective and reproducible RP-HPLC method according to the ICH guidelines for the simultaneous estimation of rosuvastatin calcium and amlodipine besylate in their combined dosage forms and for drug dissolution studies. The method involves gradient elution of drugs in a stationary phase of Luna 5µ C18 column (250 mm x 4.60 mm) using a mobile phase mixture of acetonitrile and phosphate buffer of pH 2.5 in the ratio 45:55 % v/v, with a flow rate of 1.5 ml/min in ambient temperature for separation and quantification of the drugs. The injection volume was 10µl and ultraviolet detector was set at 240 nm. Total runtime was less than 9 minutes. Under the above mentioned conditions, the system was found to elute rosuvastatin calcium at approximately 6.08 mins (Assay), 6.17 mins (dissolution) and amlodipine besylate at approximately 2.5 min (dissolution), 2.7 min (assay). Linear regression analysis data for the calibration plots showed good linear relationship with r^2= 0.993 with respect to peak area in the concentration range 8 -1.2 µg/ml for rosuvastatin and r^2= 0.996 with respect to peak area in the concentration range 4-6 µg/ml concentration of amlodipine. The percent of recovery was found to be in the range of 98-102% for both the drugs. The developed and validated assay method was found to be accurate, precise, robust and specific which allows its adoption for the routine quality control *in-vitro* dissolution studies of both the pure drug and the combination formulation.

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List of Acronyms

API = active pharmaceutical ingredient

HPLC = High Performance Liquid Chromatography

HPTLC= High Performance Thin Layer Chromatography

UPLC = Ultra Pressure Liquid Chromatography

GC = Gas Chromatography

GS-MS = Gas Chromatography-Mass Spectrometry

ICP-MS = Inductivity Coupled Plasma Mass Spectrometry

GS-IR = Gas Chromatography-Infrared Spectrometry

DSC = Differential Scanning Calorimetry

DTA = Differential Thermal Analysis.

USP = United State Pharmacopeia

ICH = International Conference on Harmonization

FT-IR = Fourier Transform Infrared Radiation

UV- ray = ultraviolet ray

HMG-CoA = 3-hydroxy-3-methylglutaryl-coenzyme A

LDL-C = low-density lipoprotein cholesterol

Total-C = total cholesterol

VLDL = very low-density lipoprotein

CAD = coronary artery disease

CCBs = calcium channel blockers

GQCLP = good quality control laboratory practice

RSD = relative standard deviation

STD = standard deviation

SOP = standard operating procedures

PDA = photo diode array

NMT = not more than

mins = minutes

mg = milligram

ml = millilitre

µg = microgram

nm = nanometer

LOD = limit of detection

LOQ = limit of quantification

THF = tetrahydrofuran

EM = electromagnetic

IR = infrared

KBr = potassium bromide

ACN = acetonitrile

Chapter 1

Introduction

Analysis is basically the study of separating, identifying and determining the relative amount of components of natural and artificial materials for characterizing it both quantitatively and qualitatively. Qualitative analysis gives an indication of the identity of the chemical species in the sample whereas quantitative analysis determines the amount of certain components in the sample. It is notable that most of the analytical tests are based on measuring specified components in the presence of a sample matrix and/or related substances and consequently isolation or separation of the target analytes preceding quantitative and qualitative analysis becomes compulsory. By using optimized separation techniques, it is possible to monitor the API (for assay), organic synthetic process impurities, and degradation products during a single determination. Chemically separations can be achieved by using chromatographic method and to a much lesser extent by electrophoresis. In chromatographic method, separation is achieved by variable distribution of different components between two dissimilar phases—a stationary phase and a mobile phase; and in electrophoresis, separations are done based on the difference in the motilities of the analytes within a conductive liquid medium subjected to an electric field. Solutes are separated based on differences in their hydrodynamic size-to-charge ratios (Scypinski, 2001). Knowing the ratio of mobility to hydrodynamic radius allows the charge, or valence, of the molecule to be determined (Actipix, 2010).

Analytical performance can be done either by instrumental method or classical method to identify and quantify compounds. Classical method ascertains the color, odor, or melting point of smaller entity for their qualitative analysis and measure weight or volume for their quantitative analysis. The separation technique under classical method includes precipitation, extraction, and distillation . In respect to the classical method, instrumental method is a newer concept to determine chemical species of organic, inorganic and biochemical analytes and has replaced classical method which enables sensitive, fast, reliable determination of small amount of complex sample. This method uses a mechanical apparatus to determine the physical properties of organic inorganic and biochemical analytes such as light absorption or emission, mass to charge ratio, fluorescence, electrode potential or conductivity for quantitative and qualitative

analysis. Therefore, the application of instrumental technique for qualitative and quantitative analysis is diverse and on account of its sophistication in analysis, it has shown its immense contribution in textile analysis, chemical analysis, food purity analysis, microbial analysis, nutritive analysis, biotechnological analysis and genetical analysis. Instrumental analysis is mainly accomplished by spectrophotometric, electrochemical, chromatographic and thermal analytical methods (Figure 1).

While developing any formulation, compatibility study of a drug with excipients must be done to support product development and improvement. A formulation is a composition containing active pharmaceutical ingredient (API) and other inactive ingredients known as excipients. To serve specific purposes of ensuring product performance, formulation must be chemically and physically stable throughout the manufacturing process and product shelf life along with their optimum bioavailability. Excipient compatibility studies are conducted to predict their possible compatibility with the target drug and justification of their usage. Therefore, while designing any new formulation studying the compatibility of single API with excipients or combined drug product with each other and excipients by various analytical techniques is imperative. An undesirable drug interaction of one or more components results in changes physical, chemical, microbiological or therapeutic properties of the dosage form (Qiu et al., 2009). Besides, if the combined dosage form is formulated, incompatibility may arise in between the two API. So the possible incompatibilities among the formulated ingredients need to be studied to select the dosage form's compatible ingredients and to establish the stability profile. The analytical testing for drug-excipient compatibility study can be done as follows:

1. Thermal method of analysis
    a) DSC- differential scanning calorimetry
    b) DTA- Differential thermal analysis.
2. FT-IR Spectroscopy
3. DFS- Diffuse reflectance spectroscopy
4. Chromatography
    a) TLC- Thin layer Chromatography
    b) SIC-Self interactive chromatography
5. Miscellaneous
    a) Fluoroscence spectroscopy

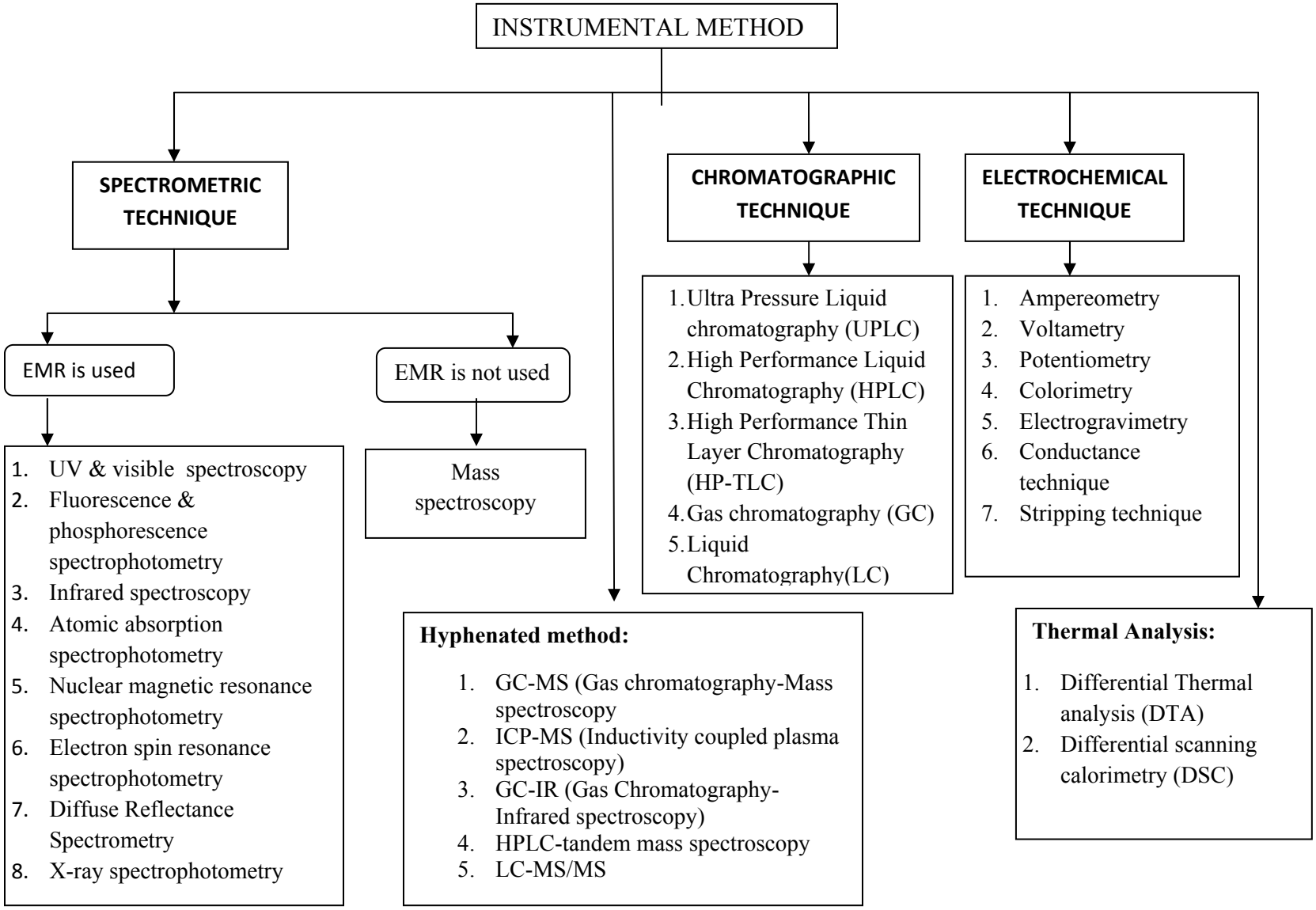


Figure-1: Classification of instrumental technique

b) Vapour pressure osmometry

The pharmaceutical products are generally formulated in specific dosage forms with the objective of delivering the drug effectively to patients. While developing any formulation different experimentation is done for the evaluation of strength, quality, purity, potency and optimum bioavailability of the API in that specific dosage form to ascertain its efficacy. Therefore, the selection of the appropriate method along with process optimization and validation of that method by changing one or more variables to assure the suitable and accurate evaluation of any product against its defined specification and quality attributes prior to the manufacture of the dosage form is necessary. Once a method is developed and validated for any particular product then that can be used for routine analysis. Method development and validation is done usually for the quality evaluation of new emerging drugs. However, sometimes changes in the method need to be done when the method remains no longer suitable for its intended use. The change may be covered by the existing validation, in which case no further validation is required or the change may result in revalidation, and in some cases, redevelopment of the method followed by validation of the new method (McPolin, 2009).

Combined dosage forms of two or more drugs have been proved useful in multiple therapies as they have better patient compliance than a single drug. It is well recognized that a single drug, even when used in maximal recommended dosage will control no more than 50% of a hypertensive population (Shaikh et al., 2010). On the other hand, the skillful use of two or more agents in combination can improve hypertension control rates to well above 80% (Shaikh et al., 2010). Physicians often have a misguided belief that blood pressure can be controlled with a single drug and demonstrate to change or to add medications in those patients whose blood pressure are not at recommended goals (Shaikh et al., 2010). Therefore, the combination drug therapy is recommended for the treatment of hypertension to allow medications of different mechanism of action to complement each other and together effectively lower blood pressure at lower than maximum dosage of each (Atram et al., 2009). Hence, the analytical chemistry has thrown challenges in developing the methods for their analysis with the help of a number of analytical techniques, which are available for the estimation of the drugs and their combination.

As the title of the project suggests, the study has used instrumental techniques for pharmaceutical analysis to evaluate the efficacy of the proposed formulated combined dosage form using a calcium channel blocker (appendix 1) & a statin (appendix 2).

For the drug-excipient compatibility study of rosuvastatin and amlodipine, FTIR testing has been done because of their sophisticated techniques in determining precisely the compatibility between the rosuvastatin calcium (appendix 3) and amlodipine besylate (appendix 4) along with their compatibility with the excipients. FT-IR, Fourier Transform Infrared Radiation, is the study of the interaction of electromagnetic radiation from the IR region of the EM spectrum (4000-400) cm^{-1} with a molecule where absorption of certain frequencies of the radiation by the atoms of the substance leads to molecular vibration (appendix 5). The frequencies of absorbed radiation are unique for each atom or group of atom, which provide the characteristics of bonds associated with a substance. Usually if incompatibility arises during FTIR study for any particular excipient, DSC (Differential Scanning Calorimetry) study, which is a thermo analytical technique, is done for further confirmation of incompatibility. Other compatibility studies for further confirmation can be conducted but was not done in the present study due to time constraints. Method development and validation of the analytical assay method of the combination formulation of rosuvastatin calcium and amlodipine besylate was then done to verify the sensitivity of detecting rosuvastatin and amlodipine in their combination tablet dosage form according to USP & ICH guidelines. *In vitro* dissolution of rosuvastatin and amlodipine containing tablets were also performed to validate the suitability of the proposed method. The process flow chart of the present study is shown in Figure 2.

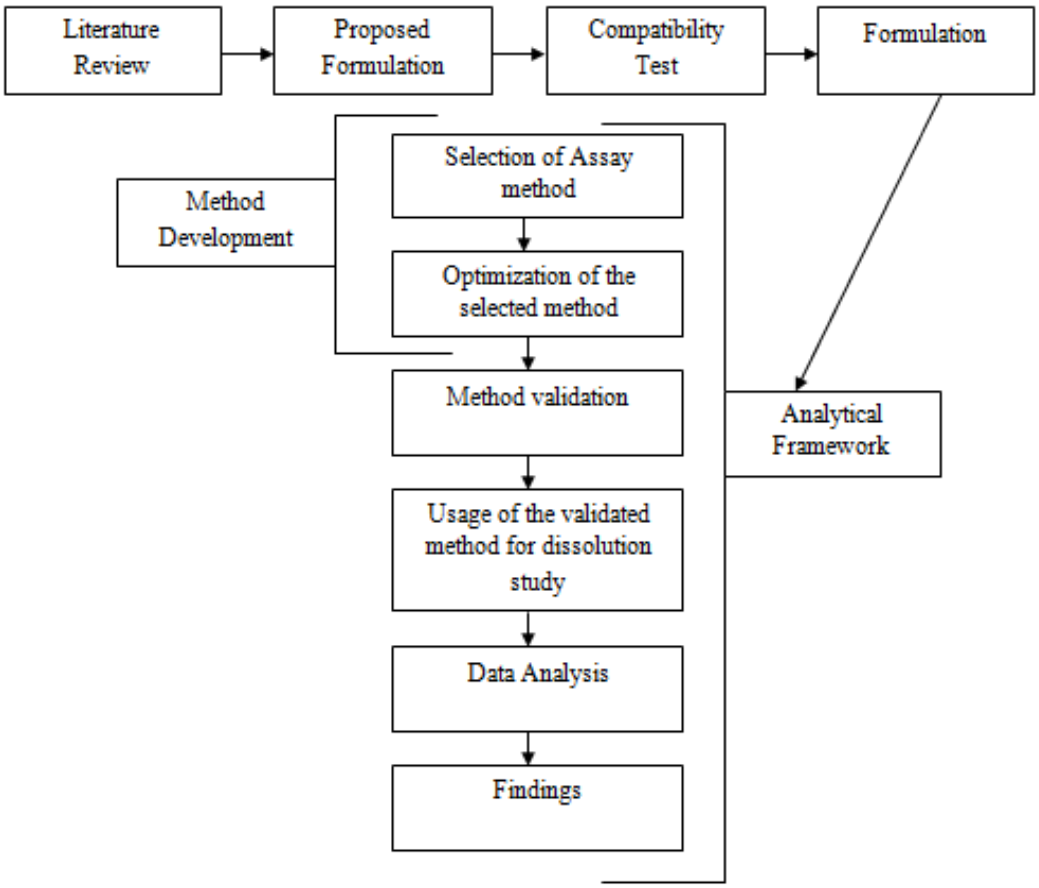


Figure-2: Flowchart of the study design of the project

1.1 Rationale of the study

Cardiovascular diseases such as coronary heart disease, cerebrovascular disease, atherothrombosis, ischemic heart disease, peripheral arterial disease are found to be prevalent among different age groups of people especially among the young generation. The current trend of fast food intake, imbalance diet control, modernization and urbanization, busy work schedule are dominating factors behind the rapid increase in cardiovascular disease. Although there have been many advances in the management of cardiovascular diseases (CVD) during the last several years, these are still the main cause for morbidity and mortality (Gowda et al., 2012). Hypertension and dyslipidemia are important, modifiable cardiovascular (CV) risk factors that frequently coexist, and together have an increase in coronary heart disease related events that may be greater than expected from the simple addition of the risk associated with each condition (Blank et al., 2005). Treatment with the combination of two or more drugs may be much effective in multiple therapies in reducing the rate of cardiovascular events than treatment with single formulation imparting monotherapies. It is well recognized that a single drug, even when used in maximal recommended dosage will control no more than 50% of a hypertensive population (Shaikh et al., 2010). On the other hand, the skillful use of two or more agents in combination can improve hypertension control rates to well above 80% (Shaikh et al., 2010). Therefore, the rational for combination therapy is to encourage the use of lower doses of drug to reduce patient's blood pressure with the goal to minimize dose dependent side effects and adverse reactions (Atram et al., 2009). Antihypertensive and lipid-lowering medications by substantially reducing blood pressure and cholesterol levels can lead to a large reduction of cardiovascular attack events.

The fixed-dose combination containing the antihypertensive agent amlodipine and the cholesterol lowering agent atorvastatin is the first combination of its kind designed to treat two risk factors for cardiovascular disease (Devabhaktuni et al., 2009). Due to the hydrophobicity of atorvastatin, it has rapid access to non hepatic tissues which results in some undesirable side effects. Although the unwanted side effects associated with combined dosage of atorvastatin and amlodipine however has been found to be reduced when rosuvastatin is used in place of atorvastatin. Rosuvastatin, another member of the drug class statin, is hydrophilic and this makes them hepatoselective. This drug may thus be considered as a substitute of atorvastatin to

formulate a new combination of drug for dose-related reduction in systolic blood pressure, diastolic blood pressure and LDL-C in patients with co-morbid hypertension and dyslipidemia.

Amlodipine is the choice of drug as an antihypertensive for the study owing to their long duration of action and comparatively higher oral bioavailability compared to the other calcium channel blockers due to their positive charge. Amlodipine is more vasoselective with lower negative inotropic effects as well as reflex tachycardia is less prominent since fluctuations in plasma levels are less pronounced with these agents (Drug information, 2003). Moreover, amlodipine has antioxidant effects, independent of calcium channel modulation, and a vasodilatory effect via the inhibition of nitric oxide release, which inhibits platelet aggregation. These pleiotropic effects of amlodipine suggest that it is more cardio protective than other non-CCB-based treatments (Park, 2014).

In order to elucidate the dissolution profiles of rosuvastatin and amlodipine, a simple, accurate, reproducible reverse phase HPLC assay method has been developed and validated and the method has been applied for the simultaneous determination of these drugs in dissolution matrix to validate the suitability of the proposed method since no systemic studies on the design and development of such a combination formulation or its *in vitro* dissolution study are currently available in literature. Thus, a simple, accurate, efficient and reproducible reverse phase HPLC method has been developed and validated for the simultaneous determination of rosuvastatin calcium & amlodipine besylate at 240 nm in combined tablet dosage form and has been applied successfully for *in vitro* dissolution studies.

1.2 Literature review

The study commenced with an extensive review of literature. The papers related to the present study were selected and information was reviewed. Several HPLC methods have been described for the determination of amlodipine when used alone (Avadhanulu, 1996; Basavaiah, 2005; Fang, 2007; Li, 2006 ; Patki, 1994; Shang, 1996, Ustun, 2006) and in combination with atorvastatin (Acharjya, 2010; Chaudhari, 2010; Freddy, 2005; Mohammadi, 2007; Rajkondawar, 2006; Shah, 2006; Sivakumar, 2007, Haritha, 2014), with rosuvastatin (Banerjee, 2013; Tajane, et al., 2012) and with olmesartan medoxomil (Patil, 2001). Similarly, a survey of the analytical literature for HPLC, UV spectrophotometric determination of rosuvastatin when used alone (Chakraborty, 2011; Babu, 2014) and in combination with

ezetimibe (Anuradha et al., 2010), amlodipine (Banerjee, 2013; Tajane et al., 2012) in pharmaceutical preparations has also been described. The HPLC method described for simultaneous determination of rosuvastatin and amlodipine in pharmaceutical preparations (Banerjee, 2013; Tajane et al., 2012) however, are not developed for *in-vitro* dissolution profile of rosuvastatin calcium and amlodipine besylate from their combination drug product and thus has not been reported in the literature.

Chapter 2

Methodology

The research methodology of this project has been developed based on a proposed combination formulation of statins with calcium channel blocker. In the study, rosuvastatin, a member of statin has been combined with calcium channel blocker amlodipine, in the amount of 10 mg and 5 mg respectively. Excipients have been chosen on the basis of the existing formulation of atorvastatin and amlodipine and their compatibility with the active ingredients has been verified. The proposed formula of the combination drug is given below (Table 1):

Table 1: Proposed Formula of the combination drug

Active Pharmaceutical Ingredients	Amount
Rosuvastatin (as Rosuvastatin calcium)	10 mg
Amlodipine (as Amlodipine besylate)	5mg
Excipient	**Justification (of use)**
Pregelatiized starch	Filler
Microcrystalline Cellulose	Binder
Sodium starch glycolate	Disintegrate
Colloidal Sillicon Dioxide	Glidant
Butylated Hydroxyanisole	Antioxidant
Magnesium stearate	Lubricant

2.1 Excipient compatibility study

While developing any formulation, excipient compatibility studies are done to select the viable excipients that are physically and chemically compatible with the API. In the present research, FT-IR study was conducted to verify the compatibility of the two APIs, rosuvastatin calcium and

amlodipine besylate with the chosen excipients. FT-IR, Fourier Transform Infrared spectroscopy is the study of the interaction of electromagnetic radiation from the IR region of the EM spectrum (4000-400) cm^{-1} with a molecule through which IR radiation is passed. The nature of interaction depends upon the functional groups present in the substance. For this purpose, fourteen FT-IR tests were done by mixing each drug entities separately with the individual excipient in the ratio of 1:1 along with separate tests of pure sample of rosuvastatin and amlodipine. The IR spectrum exhibiting the transmittance of different functional groups of the pure sample of rosuvastatin and amlodipine within 4000-400cm^{-1} region were checked, studied & recorded and their comparison had been done with the IR spectrum exhibiting transmittance of those same functional groups in presence of all the excipients individually. If the expressions of the functional groups of the pure drug entities come in similar pattern in presence of excipient as in the pure sample, the drug can be claimed compatible in presence of excipient. The tests were designed in 1:1 ratio as follows:

1. Rosuvastatin calcium (standard)
2. Rosuvastatin calcium + Pregelatinized starch
3. Rosuvastatin calcium + Microcrystalline cellulose
4. Rosuvastatin calcium + Sodium starch glycolate
5. Rosuvastatin calcium + Colloidal Sillicon dioxide
6. Rosuvastatin calcium + Butylated hydroxyanisole
7. Rosuvastatin calcium +Magnesium stearate
8. Amlodipine besylate (standard)
9. Amlodipine besylate + Pregelatinized starch
10. Amlodipine besylate + Microcrystalline cellulose
11. Amlodipine besylate + Sodium starch glycolate
12. Amlodipine besylate + Colloidal Sillicon dioxide
13. Amlodipine besylate + Butylated hydroxyanisole
14. Amlodipine besylate + Magnesium stearate

Preparation of samples for FT-IR:

1. **Rosuvastatin calcium (standard)**

Appropriate quantity of potassium bromide (KBr) and rosuvastatin calcium standard (100:1) were mixed by grinding in an agate mortar. Pellets were made with about 100 mg mixture and the FT-IR spectra were recorded with FT-IR 8400 Fourier transform Infrared spectrophotometer, Shimadzu in the range of 4000-400 cm^{-1}.

2. **Rosuvastatin calcium + Pregelatinized starch:**

Appropriate quantity of potassium bromide (KBr), rosuvastatin calcium standard and pregelatinized modified starch (100:1:1) were mixed by grinding in an agate mortar. Pellets were made with about 100 mg mixture and the FT-IR spectra were recorded with FT-IR 8400 Fourier transform infrared spectrophotometer, Shimadzu in the range of 4000-400 cm^{-1}.

3. **Rosuvastatin calcium + Microcrystalline cellulose:**

Appropriate quantity of potassium bromide (KBr), rosuvastatin calcium standard and microcrystalline cellulose (100:1:1) were mixed by grinding in an agate mortar. Pellets were made with about 100 mg mixture and the FT-IR spectra were recorded with FT-IR 8400 Fourier transform infrared spectrophotometer, Shimadzu in the range of 4000-400 cm^{-1}.

4. **Rosuvastatin calcium + Sodium starch glycolate:**

Appropriate quantity of potassium bromide (KBr), rosuvastatin calcium standard and sodium starch glycolate (100:1:1) were mixed by grinding in an agate mortar. Pellets were made with about 100 mg mixture and the FT-IR spectra were recorded with FT-IR 8400 Fourier transform infrared spectrophotometer, Shimadzu in the range of 4000-400 cm^{-1}.

5. **Rosuvastatin calcium + Colloidal Sillicon dioxide:**

Appropriate quantity of KBr potassium bromide (KBr), rosuvastatin calcium standard and colloidal sillicon dioxide (100:1:1) were mixed by grinding in an agate mortar. Pellets were made with about 100 mg mixture and the, FT-IR spectra were recorded with FT-IR 8400 Fourier transform infrared spectrophotometer, Shimadzu in the range of 4000-400 cm^{-1}.

6. **Rosuvastatin calcium + Butylated hydroxyanisole**:

Appropriate quantity of potassium bromide (KBr), rosuvastatin calcium standard and butylated hydroxyanisole (100:1:1) were mixed by grinding in an agate mortar. Pellets were made with

about 100 mg mixture and the FT-IR spectra were recorded with FT-IR 8400 Fourier transform infrared spectrophotometer, Shimadzu in the range of 4000-400 cm^{-1}.

7. Rosuvastatin calcium + Magnesium stearate:

Appropriate quantity of potassium bromide (KBr), rosuvastatin calcium standard and Magnesium stearate (100:1:1) were mixed by grinding in an agate mortar. Pellets were made with about 100 mg mixture and the FT-IR spectra were recorded with FT-IR 8400 Fourier transform infrared spectrophotometer, Shimadzu in the range of 4000-400 cm^{-1}.

8. Amlodipine besylate (standard):

Appropriate quantity of potassium bromide (KBr) and amlodipine besylate standard (100:1) were mixed by grinding in an agate mortar. Pellets were made with about 100 mg mixture and the FT-IR spectra were recorded with FT-IR 8400 Fourier transform Infrared spectrophotometer, Shimadzu in the range of 4000-400 cm^{-1}.

9. Amlodipine besylate + Pregelatinized starch:

Appropriate quantity of KBr (Potassium bromide), amlodipine besylate standard and pregelatinized starch (100:1:1) were mixed by grinding in an agate mortar. Pellets were made with about 100 mg mixture and the FT-IR spectra were recorded with FT-IR 8400 Fourier transform infrared spectrophotometer, Shimadzu in the range of 4000-400 cm^{-1}.

10. Amlodipine besylate + Microcrystalline cellulose:

Appropriate quantity of potassium bromide (KBr), amlodipine besylate standard and microcrystalline cellulose (100:1:1) were mixed by grinding in an agate mortar. Pellets were made with about 100 mg mixture and the FT-IR spectra were recorded with FT-IR 8400 Fourier transform infrared spectrophotometer, Shimadzu in the range of 4000-400 cm^{-1}.

11. Amlodipine besylate + Sodium starch glycolate:

Appropriate quantity of potassium bromide (KBr), amlodipine besylate standard and sodium starch glycolate (100:1:1) were mixed by grinding in an agate mortar. Pellets were made with about 100 mg mixture and the FT-IR spectra were recorded with FT-IR 8400 Fourier transform infrared spectrophotometer, Shimadzu in the range of 4000-400 cm^{-1}.

12. Amlodipine besylate + Colloidal Sillicon dioxide :

Appropriate quantity of potassium bromide (KBr), amlodipine besylate standard and colloidal sillicon dioxide (100:1:1) were mixed by grinding in an agate mortar. Pellets were made with about 100 mg mixture and the FT-IR spectra were recorded with FT-IR 8400 Fourier transform infrared spectrophotometer, Shimadzu in the range of 4000-400 cm^{-1}.

13. Amlodipine besylate + Butylated hydroxyanisole:

Appropriate quantity of potassium bromide (KBr), amlodipine besylate standard and butylated hydroxyanisole (in the ratio 100:1:1) were mixed by grinding in an agate mortar. Pellets were made with about 100 mg mixture and the FT-IR spectra were recorded with FT-IR 8400 Fourier transform infrared spectrophotometer, Shimadzu in the range of 4000-400 cm^{-1}.

14. Amlodipine besylate + Magnesium stearate:

Appropriate quantity of potassium bromide (KBr), amlodipine besylate standard and Magnesium stearate (100:1:1) were mixed by grinding in an agate mortar. Pellets were made with about 100 mg mixture and the FT-IR spectra were recorded with FT-IR 8400 Fourier transform infrared spectrophotometer, Shimadzu in the range of 4000-400 cm^{-1}.

2.2. Method development & validation

A method should be developed with a goal to rapidly test preclinical samples, formulation prototypes, and commercial samples (Breaux et al., 2003*)*.The Good Quality Control Laboratory Practice (GQCLP) requires test methods to assess the compliance of pharmaceutical product with established specification and to meet proper standard of accuracy and reliability. The validated method will give consistent and reliable results which are mainly concerned with source of errors and their estimation in the experiment. If the estimated errors are within the acceptable limit, then the method is said to be validated and qualified for its intended use.

For good quality control laboratory practice, numerous methods need to be developed to ascertain the identity, claimed potency, strength, quality and purity of different drug substance and drug product. These physicochemical properties of any drug substance or others are checked through different test methods such as assay test, content uniformity test, dissolution/disintegration tests, and moisture quantity test etc. These test methods vary from one API to another. Therefore, before manufacturing or launching any new product to the market,

different test methods specific to the product need to be fixed initially so that the physicochemical properties of that drug product could be checked whenever needed to ensure the safety and efficacy throughout the shelf life including storage, distribution and use (Patil et al., 2001).

In the present study a simple, sensitive and reproducible analytical assay method with better detection range for the estimation of rosuvastatin & amlodipine in pure form and in its pharmaceutical dosage forms was developed and validated. Based on the developed and validated RP-HPLC (appendix 6) method for the assay studies, the method was further used to evaluate the in vitro dissolution study (appendix 7) of the formulated dosage form and its comparison had been done with the separate market preparations of rosuvastatin and amlodipine since combined formulation of them is not currently available in the market. For this purpose, pure sample of rosuvastatin & amlodipine, available market tablets of rosuvastatin and amlodipine and the combination formulation (proposed) of rosuvastatin and amlodipine (CF-RA) were collected in the initial phase of the study to develop the intended assay method by using RP-HPLC. A system of documentation relating to the study was also recorded & maintained from the very beginning of the study. The chemical used as reagents and the apparatus used for the studies have been listed below (Table 2 and Table 3):

Table 2: List of chemicals used

Name	Manufacturer
Acetonitrile	Active Fine Chemicals Ltd, Bangladesh
Potassium Dihydrogen Phosphate	Scarlab, Spain
Orthophosphoric Acid	ACI Labscan, RCI Labscan limited, Thailand.

Table 3: List of apparatus used

Name	Manufacturer	Model
Electronic Balance	Shimadzu, Japan	ATY-224
Ultrasonic water bath	Lab Tech, Korea	LUC-405
High Pressure Liquid Chromatography (HPLC)	Shimadzu, Japan	Prominence

Some random steps taken during method development of the combined formulation of rosuvastatin and amlodipine are been discussed below:

A. Separation technique:

Separation of rosuvastatin and amlodipine out of any sample prior to its quantitative or qualitative analysis is essential and this separation should be within the acceptable range. Therefore, to determine whether that separation is optimum for any particular study, some criteria along with its acceptable ranges had been set which may differ according to instrument type, detector, column type, dimensions, and alternative column, filter type, etc. In the present study, separation of the API has been done by HPLC. Some recommended criteria's with their acceptable separation range have been given below (Table 4).

B. Solution preparation:

To prepare solution of standards and samples of rosuvastatin and amlodipine for separation and identification the following factors were considered and documented:

a. Weighing of optimum amount of sample.
b. Requirement for dilution or buffering of solution.
c. The compatibility of diluents with the mobile phase for better baseline peak.

Table 4: Separation Criteria

Criteria	Comment
Resolution	Precise and rugged quantitative analysis requires that resolution must be greater than 1.5.
Separation time	<5-10 minutes is desirable for routine procedure (e.g. dissolution profile).
Quantification	<2% RSD for assays.
Pump pressure	<150 bar is desirable. <200 bar is usually essential (for UPLC – water and RRLC-agilent these values are 5 fold and 3 fold respectively).
Peak height	Narrows peaks are desirable for large signal/noise ratio.
Solvent consumption	Minimum mobile phase use per run is desirable.

C. Instrumental setup and separation condition:

a. The installation and operational performance of instrumentation was structured according to the laboratory standard operating procedures (SOP).

b. Before the initiation of methodology development in HPLC completely new column, solvent, diluents, filter and syringe were used in order to avoid any error which may stall the accuracy of result obtained.

c. Analysis was done using analytical condition described in secondary literatures. The method sensitivity requirements for a proposed new method are influenced by several factors. These include the instrument detection limits, method quantification limits, and the regulatory requirements for the proposed applications (RCRA program).

d. The important criteria considered for method development are resolution, sensitivity, precision, accuracy, limit of detection, limit of quantification, linearity, reproducibility, and time of analysis and robustness of the method. In all of these, the column quality plays an important role since the peak shape affects all criteria required for optimum

separation. Column dimensions and particle size affect the speed of analysis, resolution, column backpressure, detection limit, and solvent consumption.

e. Chromatography also requires a proper balance of the intermolecular forces between the analyte, the mobile phase, and the stationary phase for effective analysis.

During the HPLC/UPLC method development, the first sample was injected to assure that the selected wavelength will sense all sample components of interest (Snyder et al., 2012). Normally variable wavelength UV detector is the first choice of the chromatographers, because of their convenience and applicability for most organic samples. Here, in the study, UV spectra were obtained by PDA detector.

Due to the relatively nonpolar properties of amlodipine and rosuvastatin, a reversed phase HPLC system was used to analyze both compounds with a sufficient separation and fine peak shapes. Therefore, all the experiments were carried out on a Luna 5µ C18 column (250 mm x 4.60 mm) using different conditions of various mobile phases systematically.

D. Choice of Method:

For the estimation method of rosuvastatin and amlodipine, methods from various papers were reviewed and the preferable methodology was eventually adopted and modified after undertaking several trial and error steps. The mobile phase systems that were initially fixed focusing on the gradient elution of rosuvastatin and amlodipine are as follows:

i. Phosphate buffer (pH 2.5): Acetonitrile in the ratio 55:45 % v/v

ii. Acetonitrile: THF: water at pH 3 in the ratio 68:12:20 % v/v

E. Optimization:

After determining that the chosen analytical approach would work for its intended application with appropriate sensitivity, the general procedure is to optimize the method. During optimization one parameter is changed at a time and other conditions are isolated. The initial parameters are chosen according to the analyst's best judgment. These are then varied systematically to obtain the greatest response, least interference, greatest repeatability, etc. Developers must determine those variables which should not be changed without adversely affecting method performance (RCRA Program). Accordingly, documentation was done for each and every step.

According to (Tajane et al, 2012), the ratio of the mobile phase (Acetonitrile: THF: water at pH 3 in the ratio 68:12:20 % v/v) gave the most optimum response with least interference. Therefore, at the initial point of the study, for the selection of mobile phase, the various compositions of mobile phase verification were carried out based on the study by Tajane et al. for the gradient elution of rosuvastatin and amlodipine are mentioned as follows:

MP (1) - acetonitrile: THF: water pH 3 (68:12:20 % v/v)
MP (2) - acetonitrile: THF: water pH 3 (48:12:40 % v/v)
MP (3) - acetonitrile: THF: water pH 3 (38:12:50 % v/v)
MP (4) - acetonitrile: THF: water pH 3 (78:12:10 % v/v)
MP (5) - acetonitrile: THF: water pH 3 (58:12:30 % v/v)
MP (6) - acetonitrile: THF: water pH 3 (48:22:30 % v/v)
MP (7) - acetonitrile: THF: water pH 3 (53:17:30 % v/v)
MP (8) - acetonitrile: THF: water pH 3.5 (50:10:40 % v/v)
MP (9) - acetonitrile: THF: water pH 4 (50:10: 40 % v/v)
MP (10) - acetonitrile: THF: water pH 3 (50:10:40 % v/v)

At the initial phase of the study mobile phase containing acetonitrile: THF: water pH 3.5 in (50:10:40 % v/v) had been selected to conduct the study as it gave sharp, completely resolved peak of standard rosuvastatin and amlodipine but when the dissolution profile of market preparation of rosuvastatin was studied, the chromatogram of rosuvastatin and its symmetry were found to be unacceptable. This was one of the reasons why this particular mobile phase system was discarded, the other reason being the toxicity of THF and their detrimental effect after its disposal to the environment. Therefore, based on several considerations, the mobile phase containing acetonitrile and phosphate buffer was finally selected in the ratio of 45% and 55% respectively since it was found to give the best resolution for both the drugs.

Moreover, the sensitivity of HPLC that uses UV detection depends upon the proper selection of detection wavelength. An ideal wavelength is one that gives good response for the drugs that are to be detected. For good detection, optimization of wavelength was done at different wavelength by preparing 10µg/ml of RSV and 5µg/ml of AML. The suitable wavelength for detection of rosuvastatin calcium and amlodipine besylate was selected from the overlain spectrum of rosuvastatin and amlodipine and the selected wavelength was 240 nm.

After the initial experiments, the optimum conditions (Table 5) were found to be the mobile phase of acetonitrile: phosphate buffer (pH 2.5) and (45:55) % v/v mixture pumped at 1.5 ml/min flow rate and 240 nm UV detection wavelength. Under the optimum conditions, amlodipine and rosuvastatin were eluted at 2.7 min and 6.08 min, respectively.

F. Method Validation:

Once a method is developed, it needs to be validated. Analytical method validation is a process of establishing documented evidence that provides a high degree of assurance that a specific method and the ancillary instruments included in the method will yield consistent results which accurately will reflect the quality of the product and reliability of the test. However, changes may occur which make it necessary to evaluate whether the method is still suitability for its intended use (McPolin, 2009). The change may be covered by the existing validation, in which case no further validation is required or the change my result in revalidation and in some cases redevelopment is required followed by validation of the new method (McPolin, 2009). This will also demonstrate in a laboratory study that the performance characteristics of a method of analysis make it fit for the intended analytical application. Methods should be validated to include consideration of characteristics included in the International Conference on Harmonization (ICH) guidelines addressing the validation of analytical methods (Step-by-Step Analytical Methods Validation). It specifies the type of tests required and the order in which the tests should be conducted.

To outline the validation procedure of dissolution sample of combined formulation of rosuvastatin (10 mg) and amlodipine (5 mg) the following validation parameters was studied-

- System suitability test
- Accuracy
- Precision
- Linearity and range
- Limit of Quatitation
- Limit of detection
- Robustness
- Ruggedness

Table 5: Specified Chromatographic condition for assay method

Chromatographic Mode	Chromatographic condition
Mobile phase	Acetonitrile : Phosphate buffer = (45: 55) % v/v
Stationary phase	Luna 5µ C18 column (250 mm x 4.60 mm)
Temperature	ambient
Sample size	10µl
Flow rate	1.5 ml/min
Detection wavelength	240 nm
Total run time	8 min (approximately)
Retention time	**Rosuvastatin calcium**: Approximately 6.08 mins **Amlodipine besylate**: Approximately 2.7 min

G. Preparation of Solutions:

a) Preparation of Buffer:

About 4.0827 gm of potassium dihydrogen phosphate was dissolved in 900 ml of distilled water and the pH adjusted at 2.5 by orthosphosphoric acid. The volume was then made up to 1000 ml.

b) Preparation of Mobile Phase:

Phosphate buffer solution of pH 2.5 was mixed with acetonitrile at a ratio of 55:45. It was filtered using the filter pore size not greater than 0.45 µm. Finally the mixture was degassed in an ultrasonic bath.

c) Preparation of Diluents :

Mobile phase was used as diluents.

d) Standard Preparation:

Standard stock solution of rosuvastatin and amlodipine was prepared by dissolving 25 mg rosuvastatin calcium and 12.5 mg amlodipine besylate respectively with a small quantity of mobile phase into a clean dry 100 ml volumetric flask. It was then sonicated for 20 min and the final volume of the solution was then made up to 100 ml with mobile phase. 4 ml solution was taken into 100 ml volumetric flask to obtain a concentration of 10 µg/ml rosuvastatin and 5 µg/ml amlodipine.

e) Sample preparation:

A total of 20 tablets were accurately weighed and powdered in a clean dry mortar. An amount equivalent to 10 mg of rosuvastatin and 5 mg of amlodipine was taken conical flask and solubilised in small quantity mobile phase with the aid of ultrasonication for 15 min. The resultant solution was then filtered through WHATMAN filter paper into a clean dry 100 ml volumetric flask and finally the volume was make upto 100 ml with mobile phase. From the solution, 1 ml was taken out into 10 ml volumetric flask and dilution was done with mobile phase to get a concentration of 10 µg/ml rosuvastatin and 5 µg/ml amlodipine. From this solution further dilutions were done and were injected into the system to get the chromatogram.

2.3. In-vitro Dissolution study

Dissolution test is generally required to evaluate the release of drug from pharmaceutical dosage form as a predictor of the in vivo performance of a drug product. For the evaluation of dissolution of combined formulation of rosuvastatin calcium and amlodipine besylate, different dissolution media has been used to ascertain their percentage of release according to the respective dissolution profile in FDA.

- **Dissolution of Rosuvastatin:**

Dissolution study of rosuvastatin was done using dissolution apparatus II (Paddle) at 50 rpm in 0.05 M sodium citrate buffer of pH 6.6 at temperature (37 ± 0.5)°C for 60 minutes.

Preparation of 0.05 M Sodium citrate buffer:

14.7 gm of trisodium citrate dehydrate and 0.65 gm citric acid monohydrate was dissolved in 1 L distilled water & pH was adjusted to 6.6 using 1 M NaOH or 1 M HCl.

Preparation of standard:

25 mg rosuvastatin of working standard was accurately weighed & transferred into a clean & dry 100 ml volumetric flask. 50 ml dissolution media was added to it and shacked vigorously for 5 minutes. If necessary, for the next few minutes sonication was done. Its volume was then adjusted up to the mark and allowed to cool in room temperature. This is solution A.

4 ml solution was taken from solution A into a clean and dry 100 ml volumetric flask and 50 ml dissolution media was added to it and shacked vigorously. Its volume was then adjusted up to the marks with the dissolution media. This is solution B. The solution was filtered through 0.2µ disk filter and vial was prepared.

Preparation of sample:

900 ml dissolution medium 0.05 M sodium citrate was poured into the dissolution vessels. Then the media was warmed to a temperature of 37 ± 0.5°C. Three tablets of CF-RA (containing 10 mg rosuvastatin and 5 mg amlodipine) and three tablets of rosuvastatin available at market (top brands in the local market) were weighed and immersed into the media, one tablet on each vessel between the paddle and the bottom. The apparatus was operated at 50 rpm for 60 min. Samples of about 10 ml had been withdrawn after 10, 20, 30, 45 and 60 min. Afterwards they were filtered through Whatman filter paper or with other equivalent filter. The filtrates were then finally filtered through 0.2µ disk filter and vials were prepared.

Procedure:

The vials containing standard and sample, both in concentrations of 10 µg/ml were then placed into the tray of auto sampler of Shimadzu HPLC and they were injected under the following chromatographic conditions.

Chromatographic system:

a) **Apparatus**: Shimadzu HPLC-prominence integrated with PDA detector
b) **Column**: Luna 5µ C18 column (250 mm x 4.60 mm)
c) **Mobile phase**: Acetonitrile : phosphate buffer = 45:55
d) **Temperature**: Ambient
e) **Flow rate**: 1.5 ml/min

f) **Load:** 10 µl

g) **Retention time:** 6.08 min (approx)

h) **Run time:** 8 min (approx)

i) Wavelength: 240 nm

- **Dissolution of amlodipine:**

Dissolution study of Amlodipine was done using dissolution apparatus II (paddle) at 75 rpm in 0.01 N HCl at temperature (37 ± 0.5)°C for 60 minutes.

Preparation of 0.01 N HCL:

0.825 ml 0.01 N HCl was dissolved in 1 L distilled water and pH was adjusted to 2.5 using 1 M HCl.

Preparation of standard:

25 mg amlodipine of working standard was accurately weighed & transferred into a clean & dry 100 ml volumetric flask. 50 ml dissolution media was added to it and shacked vigorously for 5 minutes. If necessary, for next the few minutes sonication was done. Its volume was then adjusted up to the mark and allowed to cool in room temperature. This is solution A.

4 ml from solution A was taken into a clean and dry 100 ml volumetric flask and 50 ml dissolution media was added to it and shacked vigorously. Its volume was then adjusted up to the mark with the dissolution media. This is solution B. Finally, the solution was filtered through 0.2µ disk filter and vial was prepared.

Preparation of sample:

500 ml medium 0.01 N HCl was poured into the dissolution vessels. Then the media was warmed to a temperature of 37 ± 0.5°C. Three tablets of CF-RA (containing 10 mg rosuvastatin and 5 mg amlodipine) and three tablets of amlodipine available at market (top brands in the local market) were weighed and immersed into the media, one tablet on each vessel between the paddle and the bottom. The apparatus was operated at 75 rpm for 60 min. Sample of about 10 ml had been withdrawn after 10, 20, 30, 45 and 60 min. Afterwards they were filtered through Whatman filter paper or with other equivalent filter. The filtrates were then finally filtered through 0.2µ disk filter and vials were prepared.

Procedure:

The vials containing standard and sample, both in concentrations of 10 µg/ml, were then placed into the tray of auto sampler of Shimadzu HPLC and they were injected into the system under the following chromatographic conditions.

Chromatographic system:

a) **Apparatus**: Shimadzu HPLC-prominence integrated with PDA detector
b) **Column**: Luna 5µ C18 column (250 mm x 4.60 mm)
c) **Mobile phase**: Acetonitrile : phosphate buffer = 45:55
d) **Temperature**: Ambient
e) **Flow rate**: 1.5 ml/min
f) **Load:** 10 µl
g) **Retention time:** 2.8 min (approx)
h) **Run time:** 8 min (approx)
i) **Wavelength:** 240 nm

Chapter 3

Data Analysis

3.1. FT-IR study

In the study, FT-IR 8400 Fourier transform infrared spectrophotometer was employed for ascertaining the compatibility of the excipient with the API through comparative qualitative analysis of the different functional groups of pure sample of rosuvastatin calcium (Figure 3) and amlodipine besylate (Figure 4) as well as mixed sample of those drug entities separately with all the excipients individually (Figures 5-18). The results of the study are shown below in Table 6 and Table 7.

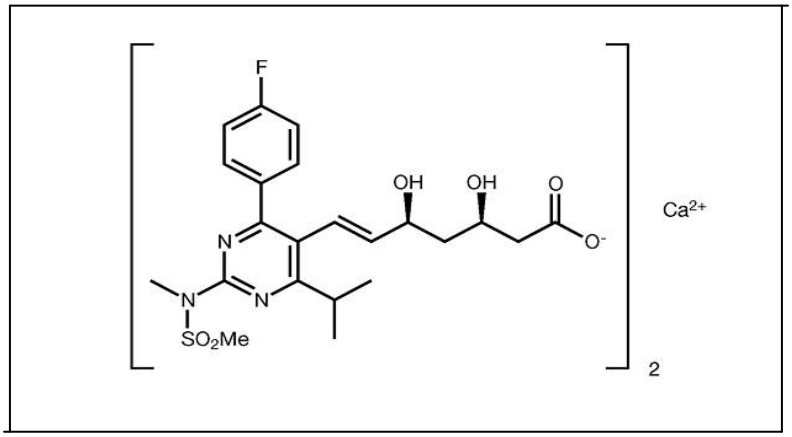


Figure 3: Structure of Rosuvastatin calcium

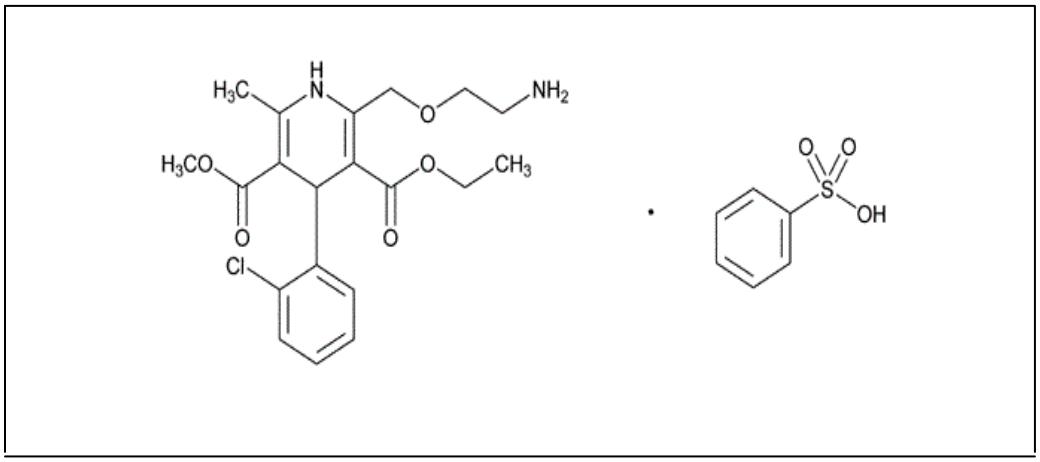


Figure 4: Structure of Amlodipine besylate

Table 6: FT-IR Study of rosuvastatin calcium (standard) and its comparison with the mixed sample of rosuvastatin calcium and individual excipients

	O-H stretching **ALCOHOL** Broad & strong 3550-3200	Dual Response 3300-2500 **O-H** stretching Carboxylic acid 3200-2700 **O-H** stretching Alcohol (intramolecular bonded)		**S=O** stretching **SULFONE** Strong 1160-1120	Remarks
Rosuvastatin calcium (standard)	3420.87	2969.55	2928.04	1156.36	
RSV + pregelatinized modified starch	3420.87	2968.55	2931.90	1155.40	Compatible
RSV + microcrystalline cellulose	3420.87	2966.62	2930.93	1156.36	Compatible
RSV + Sodium starch glycolate	3440.16	2968.55	2930.93	1155.40	Compatible Due to the presence of huge number of –OH group in Starch molecule, they together with –OH group of RSV have given common broaded response near 3400 cm^{-1} region. So, the position of the peak of –OH group is slightly diverted.
RSV + Colloidal SiO_2	3433.41	2969.51	2934.79	1113.93	Compatible The sulfone group gave out a merged peak with Si=O near to 1111 cm^{-1} region which is broaded. So the position of the peak got diverted.
RSV + Butylated hydroxyanisole	3421.83	2952.15	2915.5	1156.36	Compatible
RSV + Magnesium stearate	There was a possibility of peak but the instrument printed out the default one	2956.97	2916.47	1156.36	Compatible

Table 7: FT-IR Study of amlodipine besylate (standard) and its comparison with the mixed sample of amlodipine besylate and individual excipient

	N-H stretching Medium **Primary Amine** 3330-3250	N-H stretching Medium **Secondary Amine** 3350-3310	C-H stretching Strong **Alkene** 3100-3000	C=O stretching Strong **α,β-unsaturated ester** 1730-1715	S=O Stretching Strong **Sulfone** 1160-1120	Remarks
Amlodipine besylate (standard)	3300.31	3157.58	3069.81	1696.45	1125.5	Compatible
AMD besylate + pregelatinized modified starch	3285.85	3155.65	3066.92	1696.45	1125.5	Compatible
AMD besylate + Microcrystalline cellulose	3420.91	3169.15	Due to instrumental error the response of alkene cannot get detected. The pattern near 3000 cm^{-1} show there is a possibility of alkene response.	1696.45	1125.5	Compatible Due to the presence of huge number of –OH group,they together with the N-H group has given common broaded peak near 3300-3500 cm^{-1} region. So, the position of the peak of N-H is slightly diverted
AMD besylate + Sodium starch glycolate	3291.63	3155.65	3083.31	1696.45	1125.50	Compatible
AMD besylate + Colloidal SiO_2	The instrument printed out the default one, but there is a peak of similar pattern near 3300 cm^{-1} region	The instrument printed out the default one, but there is a peak of similar pattern near 3155 cm^{-1} region	The instrument printed out the default one, but there is a peak of similar pattern near 3085 cm^{-1} region	1696.45	1125.5	Compatible For the conduction of experiment using FT-IR, the default mode of the IR- spectrum got printed. Still the spectrum has shown the possible response of the desired functional group.
AMD. besylate + Butylated hydroxyanisole	3329.25	3154.68	3068.85	1696.45	1125.5	Compatible
AMD besylate + Mg stearate	3292.60	3164.33	3066.92	1696.45	1125.50	Compatible

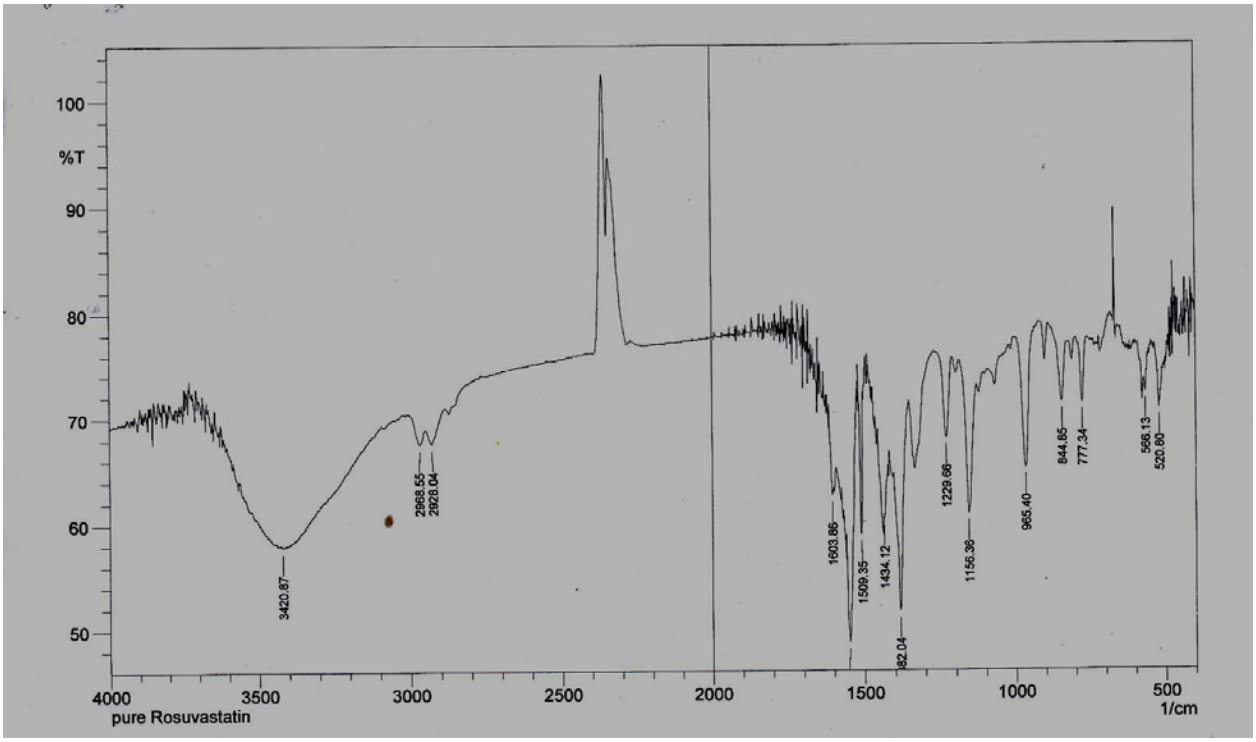


Figure 5: FT-IR study of Rosuvastatin calcium standard

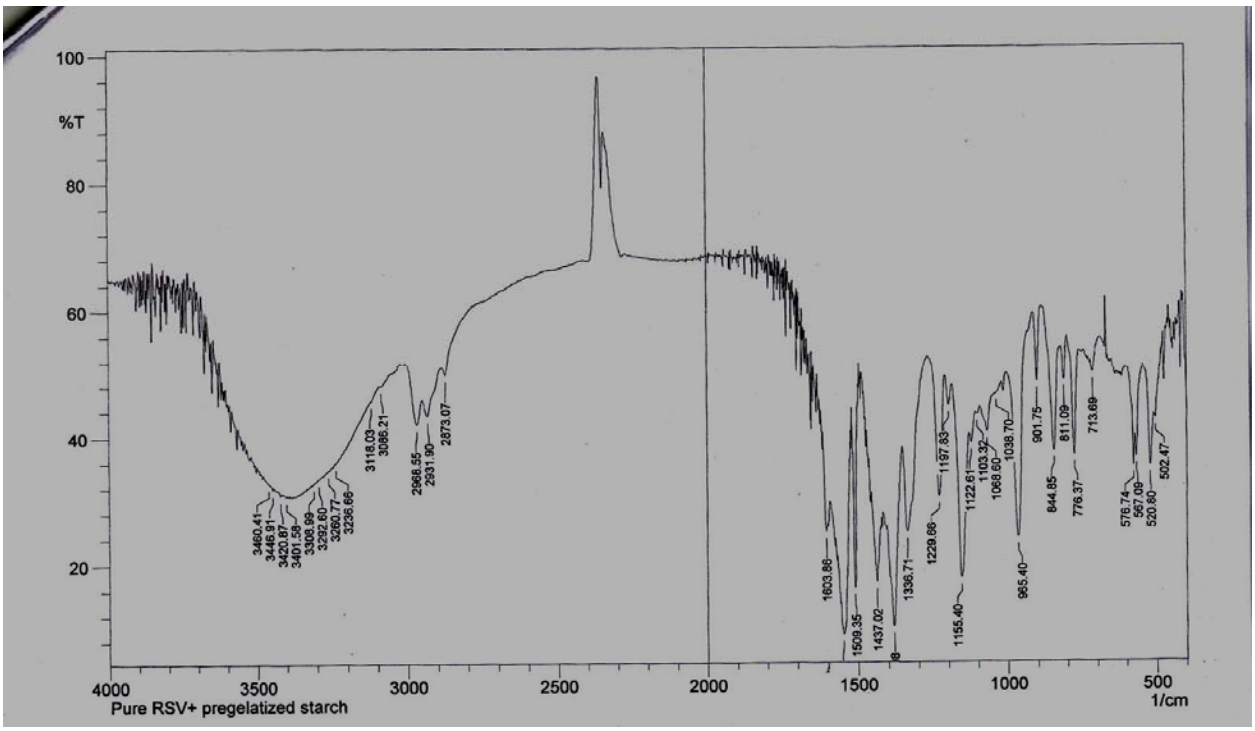


Figure 6: FT-IR study of Rosuvastatin calcium and pregelatized starch mixture (1:1)

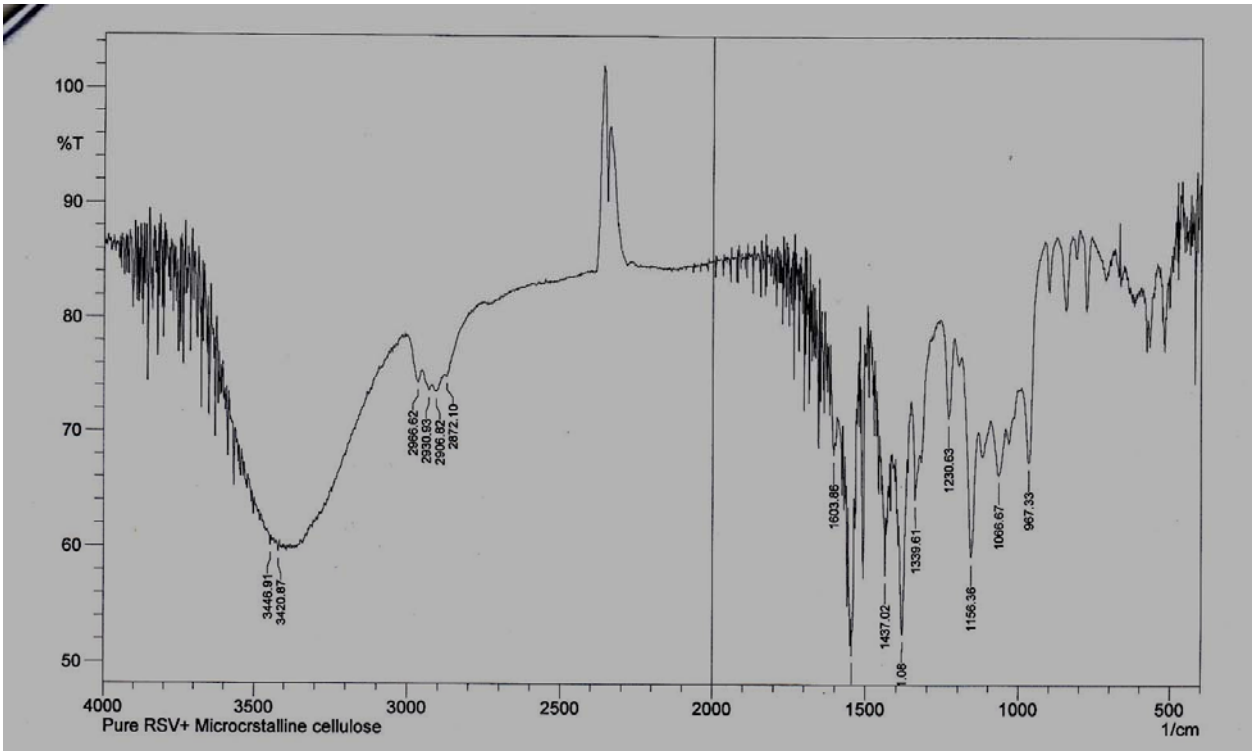


Figure 7: FT-IR study of rosuvastatin calcium and microcrystalline cellulose mixture (1:1)

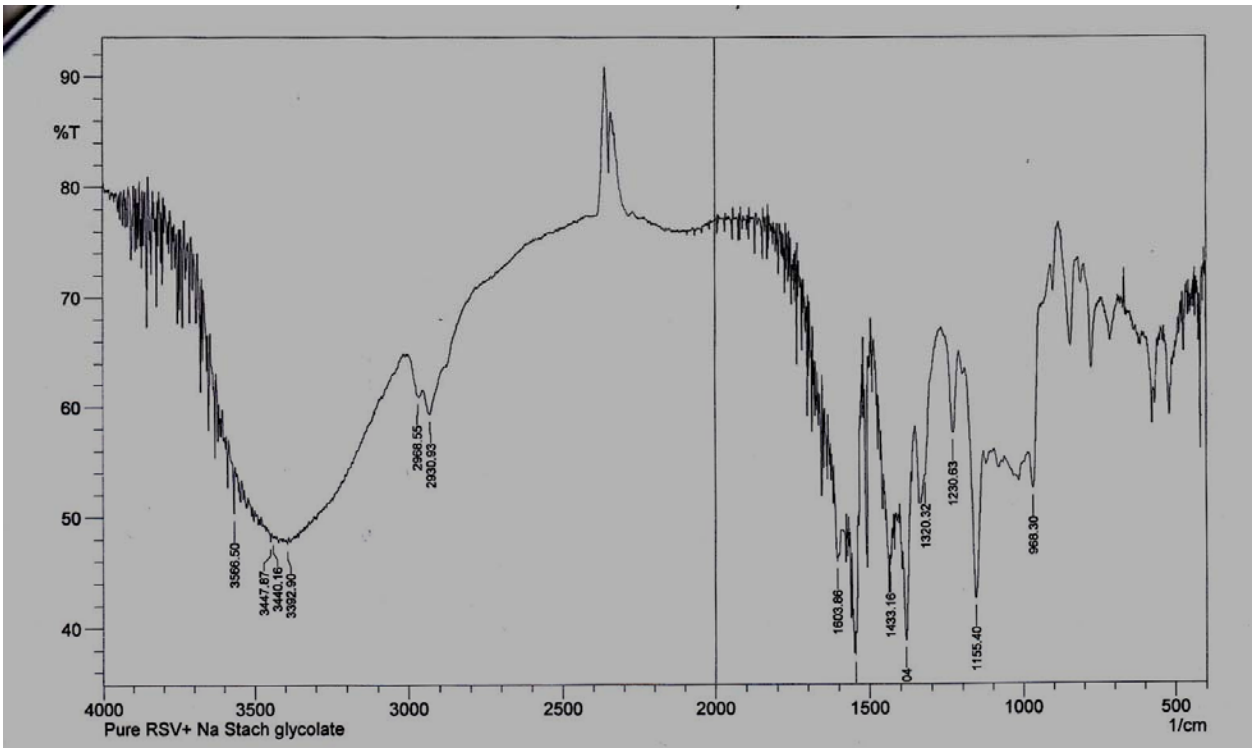


Figure 8: FT-IR study of rosuvastatin calcium and Sodium starch glycolate mixture (1:1)

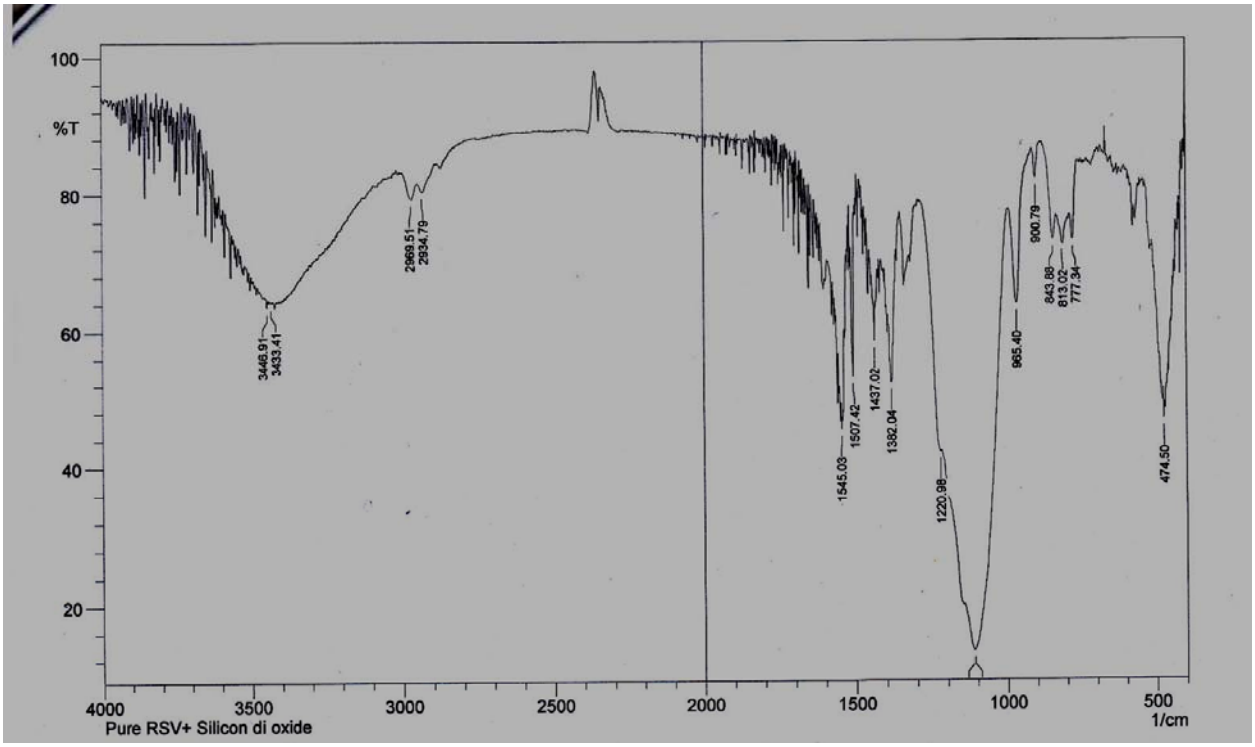


Figure 9: FT-IR study of rosuvastatin calcium and colloidal sillicon dioxide mixture (1:1)

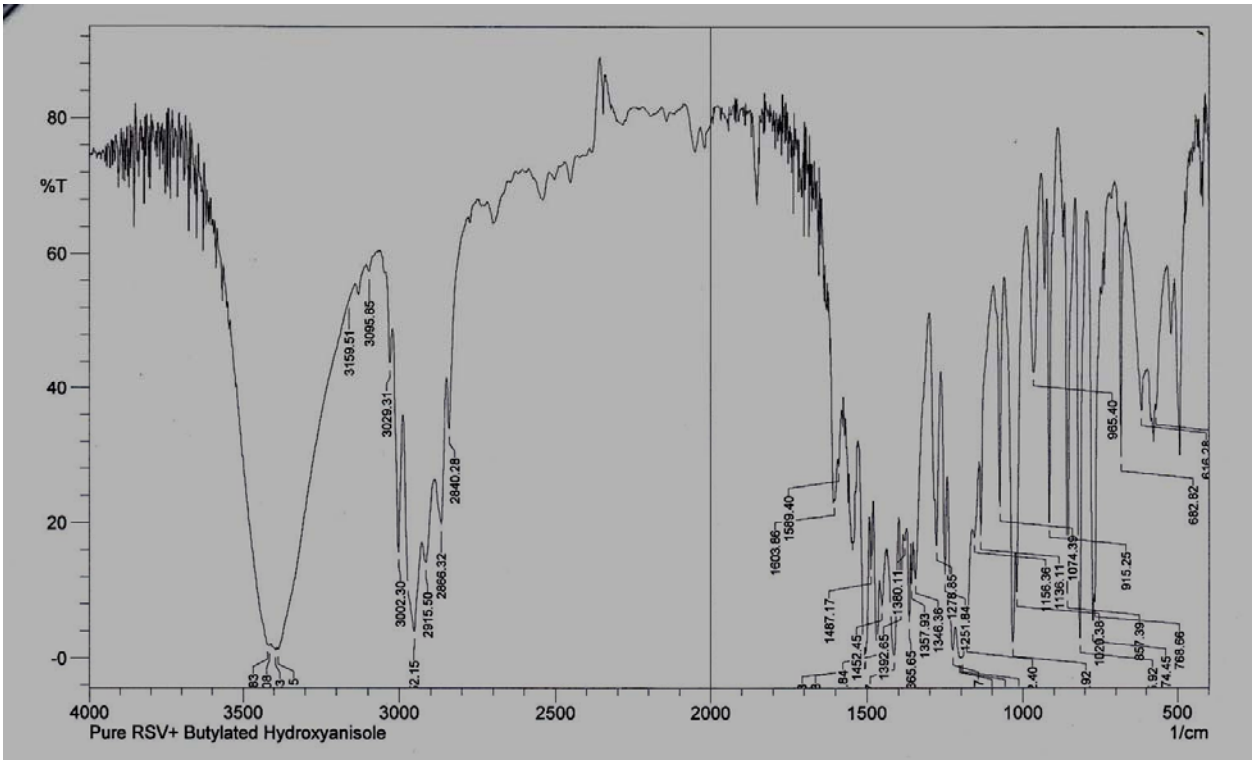


Figure 10: FT-IR study of Rosuvastatin calcium and butylated hydroxyanisole (1:1)

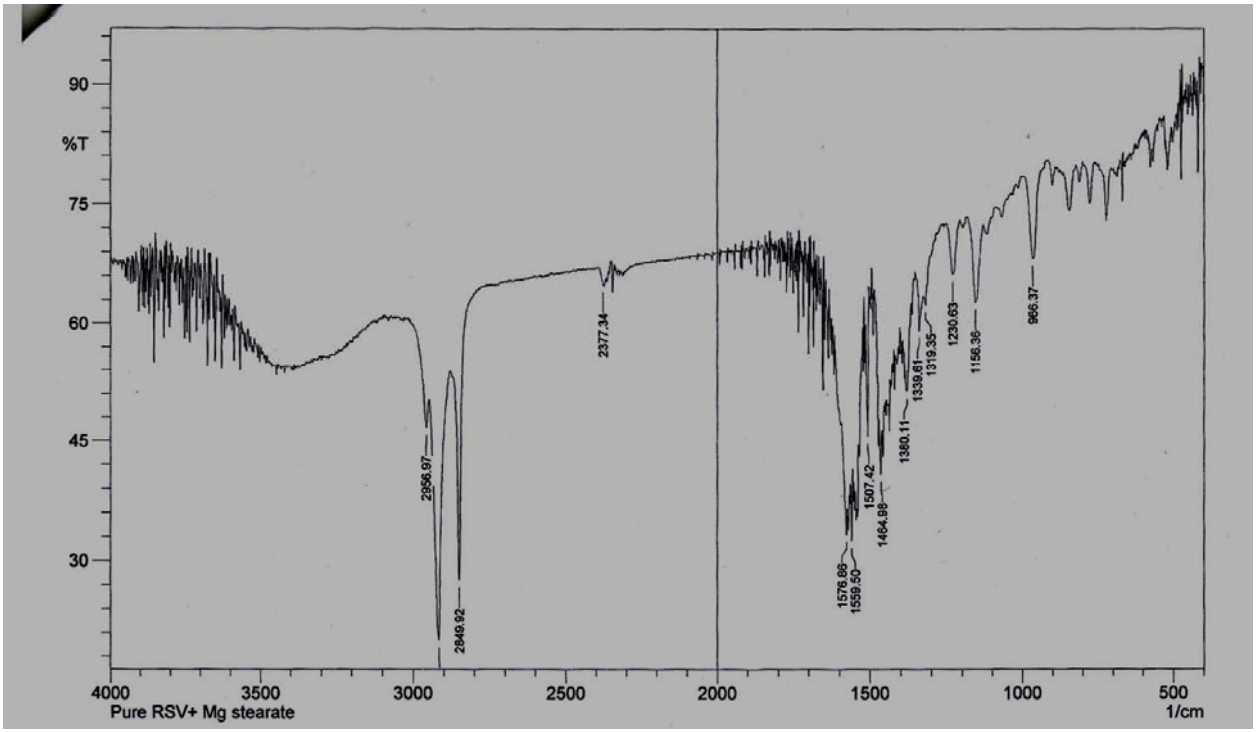


Figure 11: FT-IR study of Rosuvastatin calcium and Magnesium stearate (1:1)

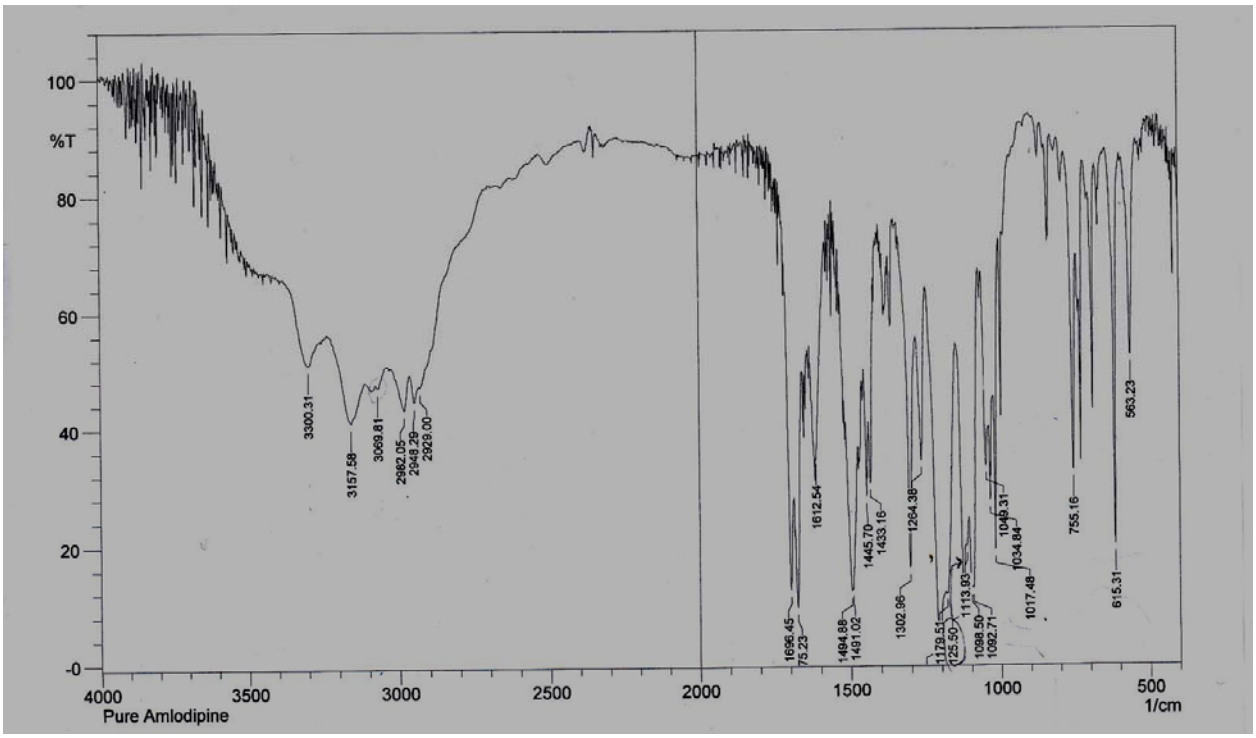


Figure 12: FT-IR study of Amlodipine besylate standard

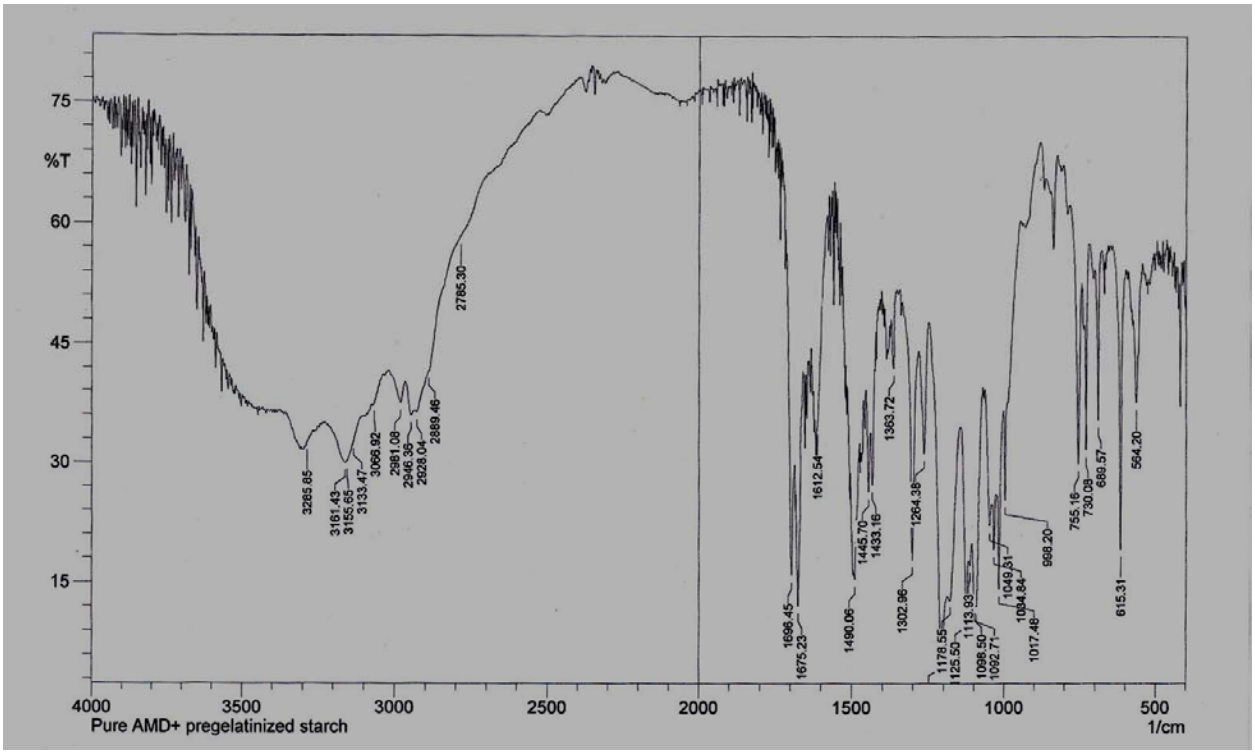


Figure 13: FT-IR study of Amlodipine besylate and pregelatized starch mixture (1:1)

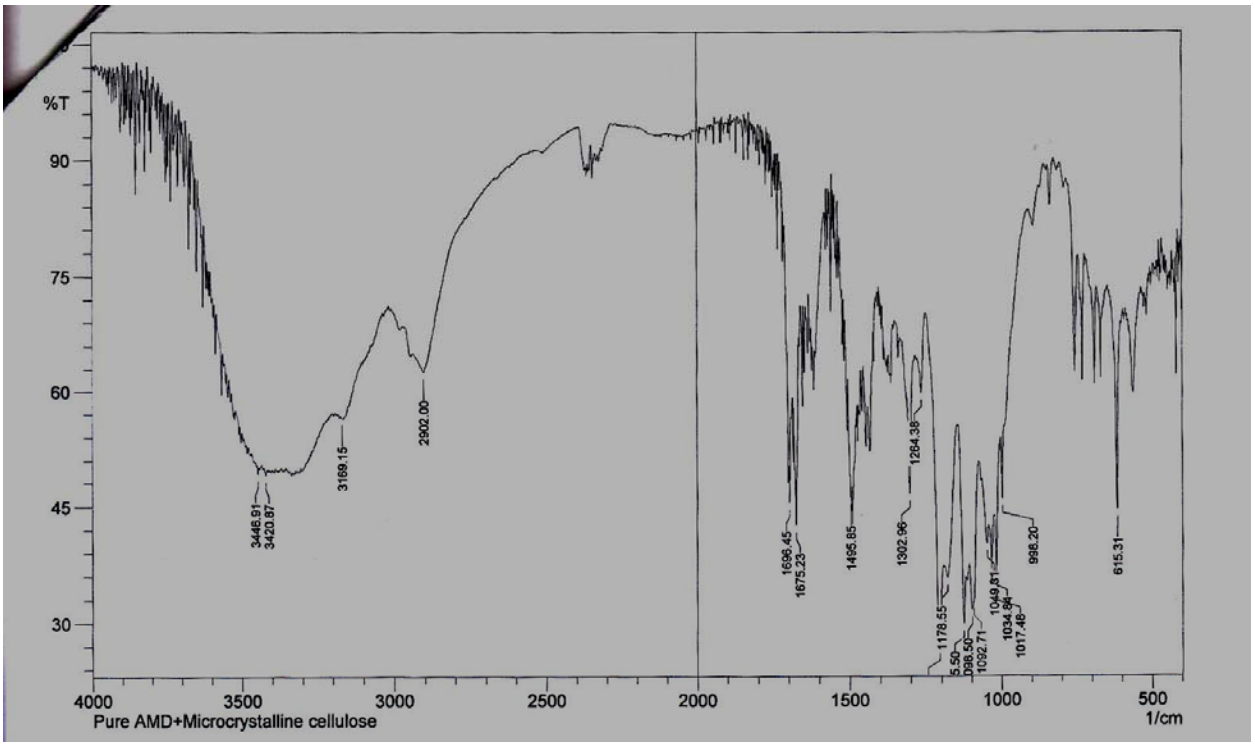


Figure 14: FT-IR study of Amlodipine besylate and microcrystalline cellulose mixture (1:1)

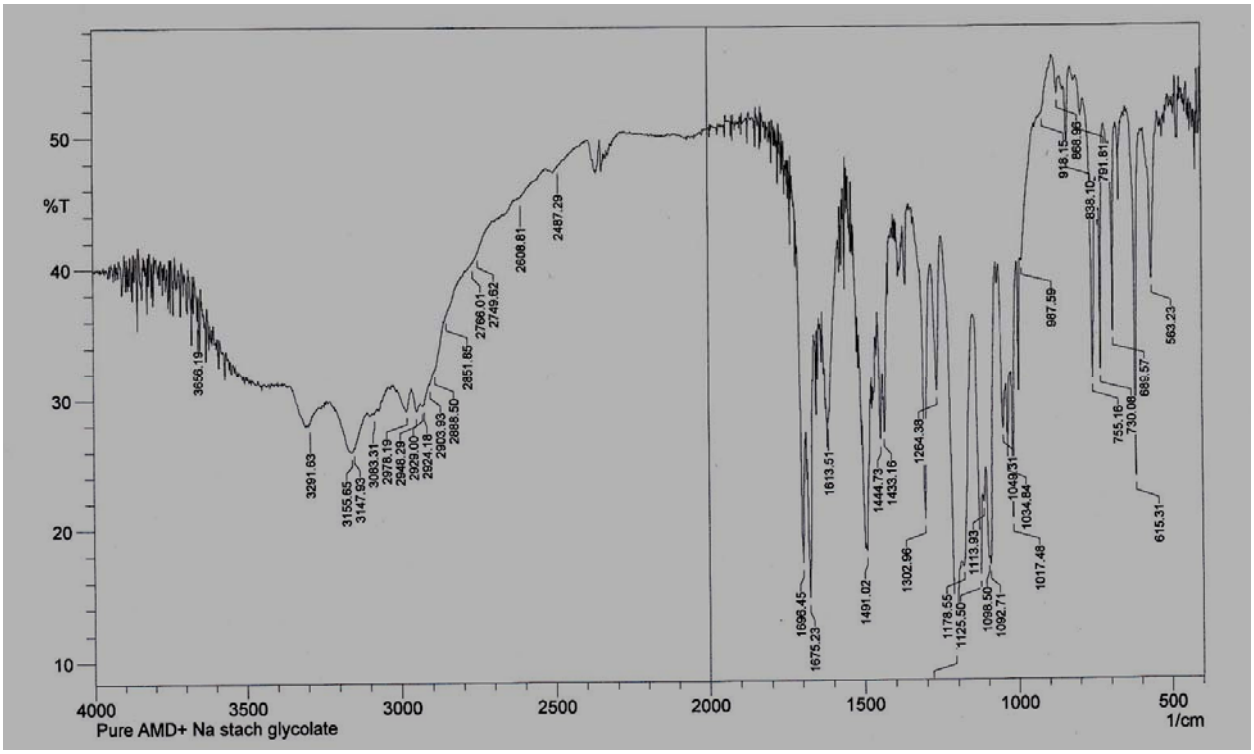


Figure 15: FT-IR study of Amlodipine besylate and Sodium starch glycolate mixture (1:1)

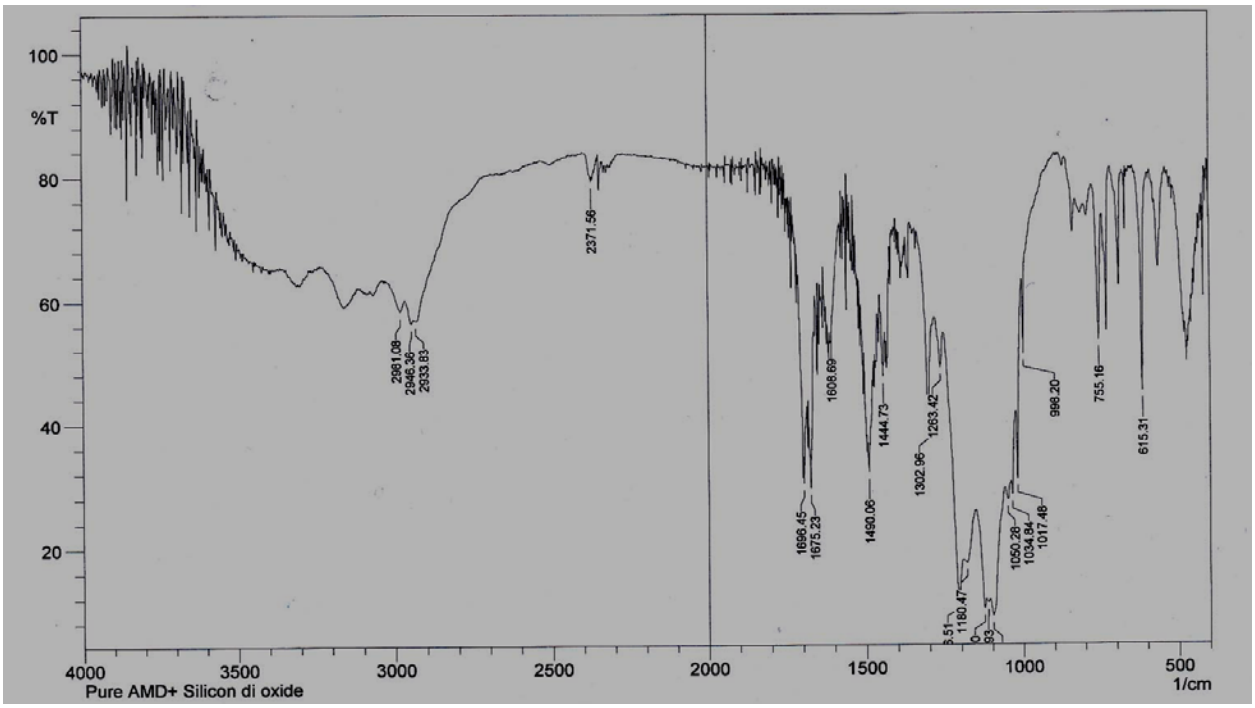


Figure 16: FT-IR study of Amlodipine besylate and colloidal sillicon dioxide mixture (1:1)

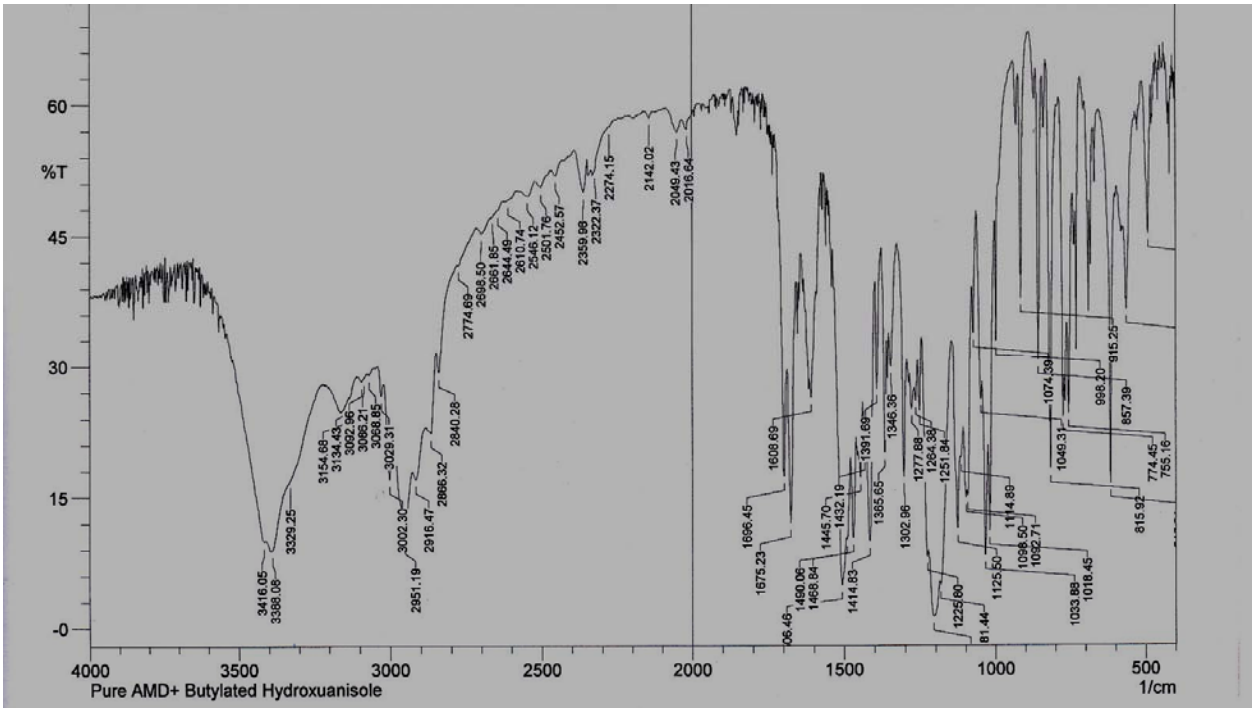


Figure 17: FT-IR study of Amlodipine besylate and butylated hydroxyanisole (1:1)

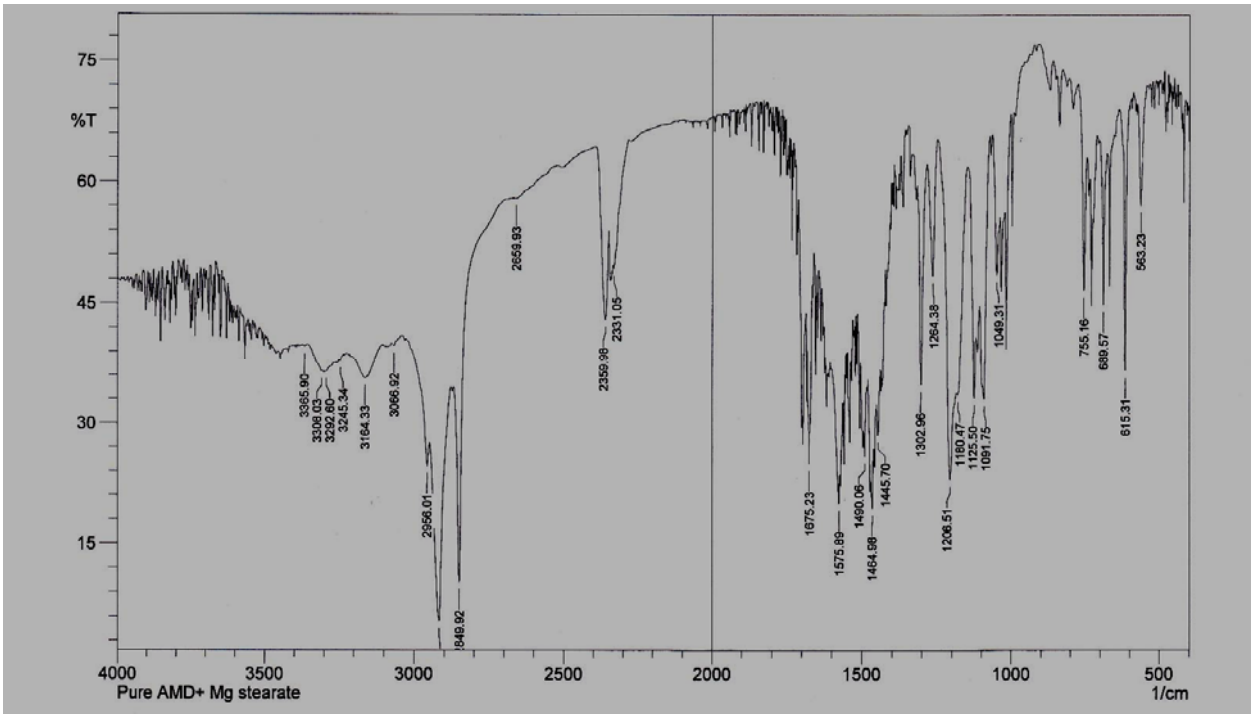


Figure 18: FT-IR study of Amlodipine besylate and Magnesium stearate (1:1)

3.2. Validation parameters for assay study

3.2.1. System Suitability Test

A suitability test was applied to the chromatograms of taken under optimum conditions to check various parameters such as column efficiency (theoretical plates), peak tailing, retention factor, and resolution (Celebier et al., 2010). Freshly prepared standard stock solution of rosuvastatin and amlodipine were injected into the chromatographic system (Figure 19) under the optimized chromatographic conditions (Patil et al., 2001). The test is considered valid if the following two considerations are met:

- The relative standard deviation for the peak area response of rosuvastatin and amlodipine for replicate injections of standard preparation is not more than 2% respectively (Qiu et al., 2009)
- Tailing factor: ≤ 2% for the rosuvastatin and amlodipine peak in standard solution.

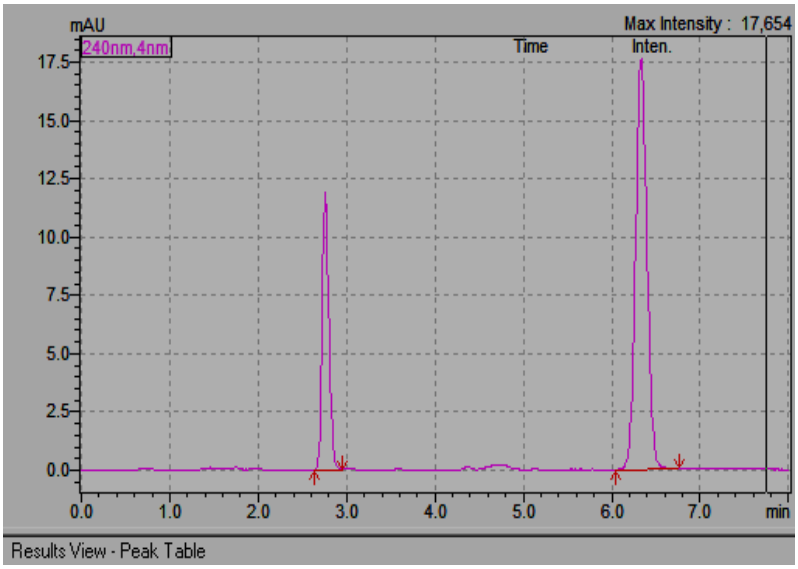


Figure 19: Chromatogram of standard Rosuvastatin calcium and amlodipine besylate

Table 8: System suitability parameters of Standard Rosuvastatin calcium

Rosuvastatin calcium				
	Tailing factor	Theoretical plate	Peak area	Retention time
1	1.165	6330	140745	6.185
2	1.163	6432	140724	6.182
3	1.123	6345	140765	6.194
4.	1.143	6349	140754	6.186
5.	1.156	6354	140798	6.18
6.	1.165	6343	140812	6.192
Average	1.153	6359	140766	6.187
STD	0.017	36.73	33.13	0.006
RSD (%)	1.45	0.578	0.024	0.089

Table 9: System suitability parameters of Standard Amlodipine besylate

Amlodipine besylate				
	Tailing factor	Theoretical plate	Peak area	Retention time
1	1.032	10751	159936	2.595
2	1.032	10702	160552	2.59
3	1.037	10754	160915	2.596
4.	1.039	10736	160468	2.593
5.	1.036	10745	160432	2.595
6.	1.033	10732	160443	2.594
Average	1.035	10737	160458	2.594
STD	0.003	18.97	313.42	0.002
RSD (%)	0.28	0.177	0.195	0.082

Data interpretation:

It is observed from the above tabulated data (Table 8 and Table 9) that the method complies with the system suitability parameters. Hence, it can be concluded that the system suitability parameters meets the requirement of method validation.

3.2.2. Linearity

Linearity is typically established by preparing solutions of the drug substance, ranging in concentration from less than the lowest expected concentration to more than the highest concentration during release (The Dissolution Procedure: Development and Validation, 2014).

Procedure:

- Samples at concentrations 80%, 90%, 100%, 110%, and 120% of the target concentration were prepared and were injected into the chromatographic condition.

- Chromatograms were taken and concentration of samples versus corresponding peak area was plotted (Table 10 and 11) to get a calibration curve (Figure 20 and 21). From the data obtained, co-relation coefficient, slope and y-intercept were calculated. Ideally, co-relation coefficient should be around 1.

Preparation of linearity samples:

Samples of different concentrations required for linearity test were prepared as follows:

- **80% solution:**

0.32 ml solution was taken from the stock solution in a 10 ml volumetric flask and volume was made up to 10 ml using mobile phase mixture.

- **90% solution:**

0.36 ml solution was taken from stock solution in a 10 ml volumetric flask and volume was made up to 10 ml using mobile phase mixture.

- **100% solution:**

0.4 ml solution was taken from stock solution in a 10 ml volumetric flaskand volume was made up to 10 ml using mobile phase mixture.

- **110% solution:**

0.44 ml solution was taken from stock solution in a 10 ml volumetric flask and volume was made up to 10 ml using mobile phase mixture.

- **120% solution:**

0.48 ml solution was taken from stock solution in a 10 ml volumetric flask and volume was made up to 10 ml using mobile phase mixture.

Table 10: Result of Linearity study of Rosuvastatin calcium

Rosuvastatin calcium		
	Concentration (mg/ml)	Peak Area
1.	0.008	125146.6
2.	0.009	143739.2
3.	0.01	162706.2
4.	0.011	173612.6
5.	0.012	191398

Table 11: Result of Linearity study of Amlodipine besylate

Amlodipine besylate		
	Concentration (mg/ml)	Peak Area
1.	0.004	50972
2.	0.0045	57631
3.	0.005	64984
4.	0.0055	69629.4
5.	0.006	76380.6

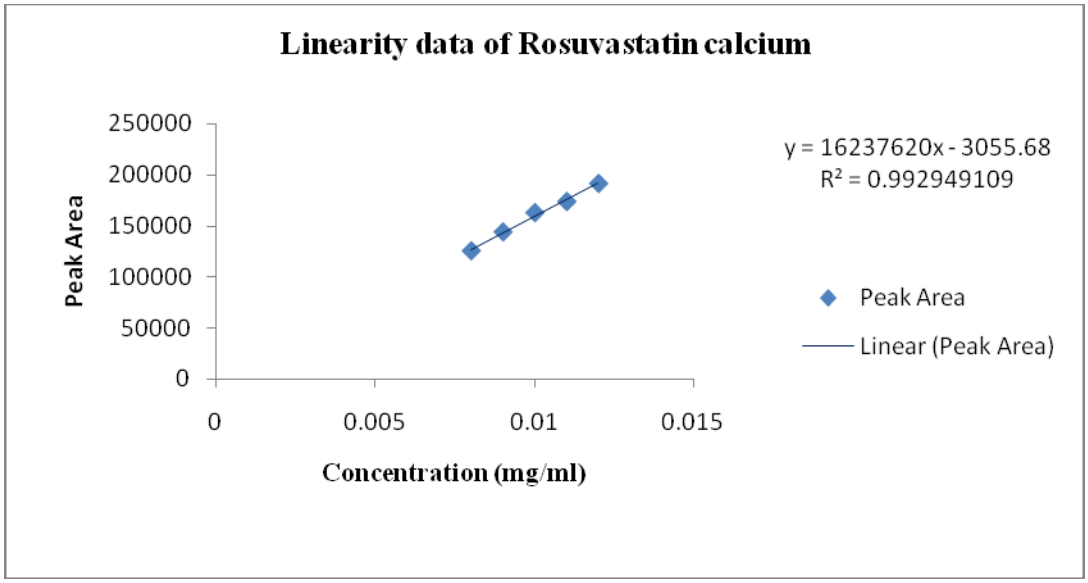


Figure 20: Linearity curve of Rosuvastatin calcium

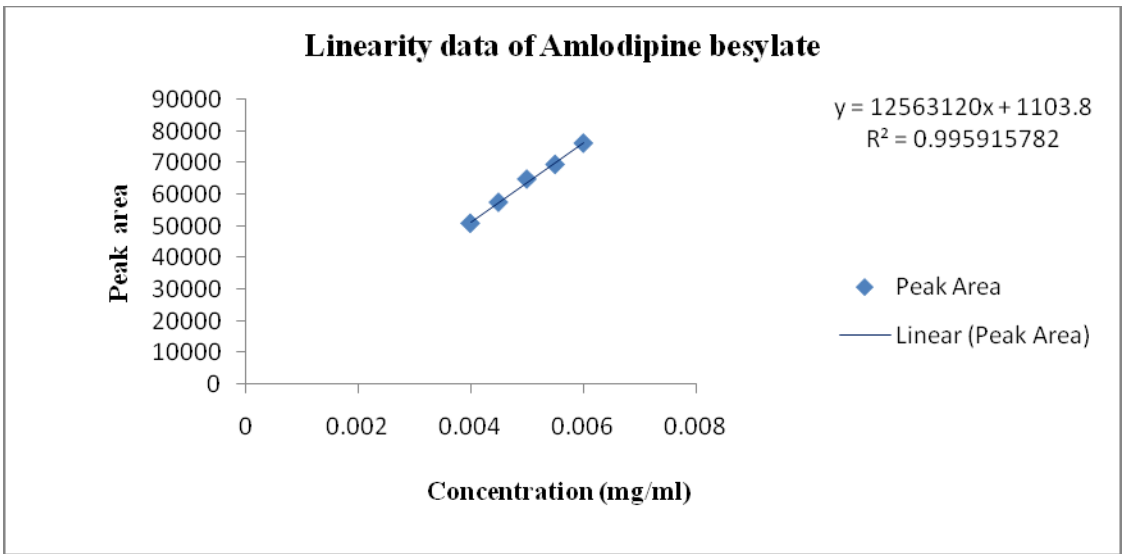


Figure 21: Linearity curve of Amlodipine besylate

Data Interpretation:

The method was found to be linear with the 4 µg/ml to 6 µg/ml concentration of amlodipine and 8 µg/ml to 1.2 µg/ml concentration of rosuvastatin. The co-relation coefficient was found to be 0.992 for Rosuvastatin and 0.995 for amlodipine.

3.2.3. Accuracy

The accuracy of the assay method was evaluated with the recovery of the standards from excipients (Tajane et al., 2012). Accuracy/recovery are typically established by preparing multiple samples containing the drug and any other constituents present in the dosage form ranging in concentration from below the lowest expected concentration to above the highest concentration during release. For this purpose, accuracy must be done on at least 3 concentrations (80%, 100% and 120%) in the expected range.

Preparation of accuracy sample:

Samples of different concentrations required for accuracy test were prepared as follows:

- **80% solution:**

3.2 ml solution was taken from the stock solution in a 10 ml volumetric flask which was previously filled with 17.5 mg placebo. The volume was made up to 10 ml using mobile phase mixture (Figure 22).

- **100% solution:**

4 ml solution was taken from the stock solution in a 10 ml volumetric flask which was previously filled with 14 mg placebo. The volume was made up to 10 ml using mobile phase mixture (Figure 23).

- **120% solution:**

4.8 ml solution was taken from the stock solution in a 10 ml volumetric flask which was previously filled with 21 mg placebo. The volume was made up to 10 ml using mobile phase mixture (Figure 24).

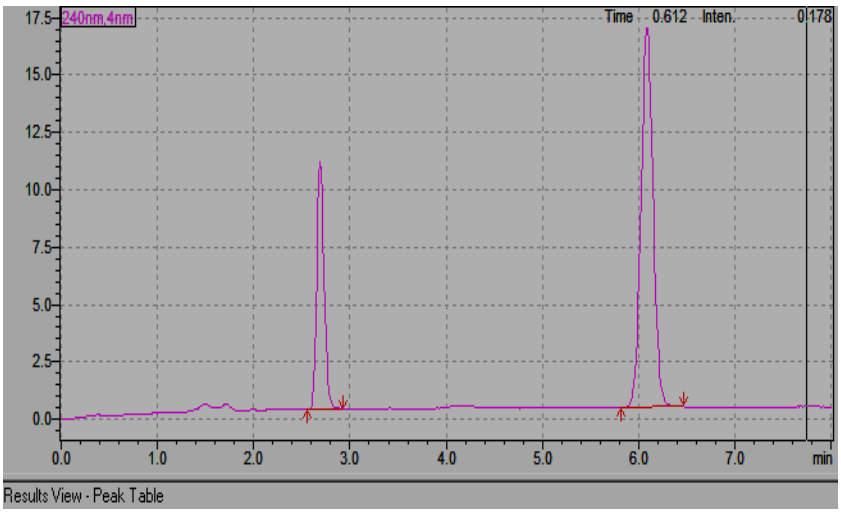


Figure 22: Chromatogram of 80% solution (accuracy)

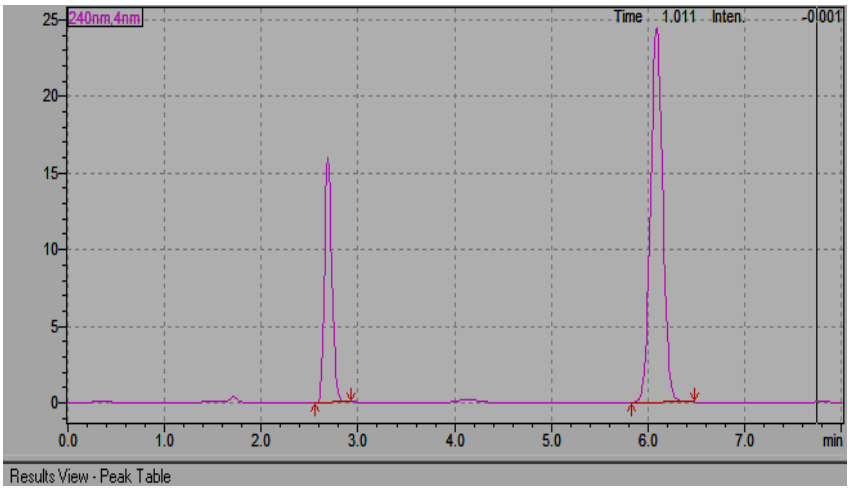


Figure 23: Chromatogram of 100% solution (accuracy)

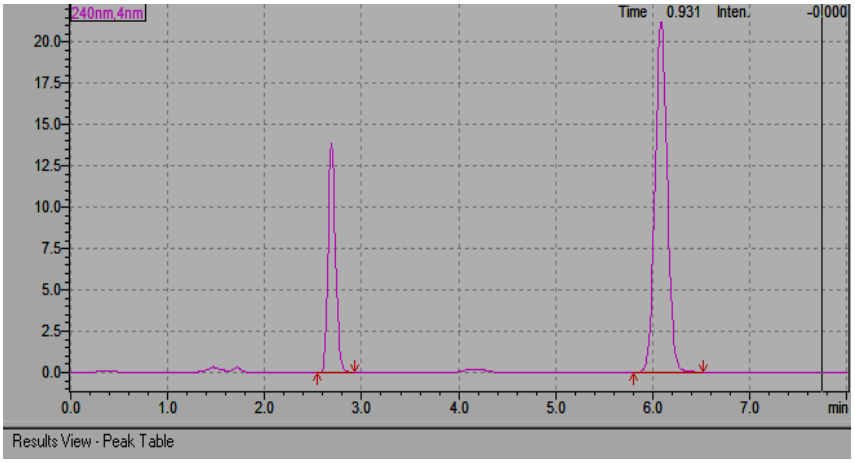


Figure 24: Chromatogram of 120% solution (accuracy)

Table 12: Result of Accuracy study of rosuvastatin calcium and amlodipine besylate

Accuracy						
	Rosuvastatin calcium			Amlodipine besylate		
Sample no.	Spike level (percentage)	Percent (%) of recovery	Mean percent (%) of recovery	Spike level (percentage)	Percent (%) of recovery	Mean percent (%) of recovery
1.	80%	99.01%	99.03%	80%	102.85%	102.88%
2.	80%	99.05%		80%	102.90%	
3.	80%	99.03%		80%	102.89%	
4.	100%	101.91%	101.9%	100%	101.86%	101.97%
5.	100%	101.89%		100%	102.09%	
6.	100%	101.90%		100%	101.97%	
7.	120%	102.04%	102.04%	120%	98.74%	98.67%
8.	120%	102.05%		120%	98.60%	
9.	120%	102.04%		120%	98.69%	

Data Interpretation:

The result of analysis (Table 12) showed excellent recoveries for both the drugs ranging from 98 % to 102% for amlodipine & rosuvastatin which suggests the accuracy of the method for the simultaneous estimation of rosuvastatin and amlodipine.

3.2.4. Precision

The precision was studied in terms of changes in peak area of standard and/or sample solution drug on the same day to evaluate the repeatability and on two different days over a period of one week to evaluate the reproducibility. The precision (percentage relative standard deviation, %RSD) was expressed with respect to the interday (Figure 26) and intra-day (Figure 27) variation in the expected drug concentration (Banerjee et al., 2013) and both the results have been compared with the standard stock solution (Figure 25).

Preparation of precision sample:

0.4 ml solution was taken from stock solution in a 10 ml volumetric flask and volume was made up to 10 ml using mobile phase mixture.

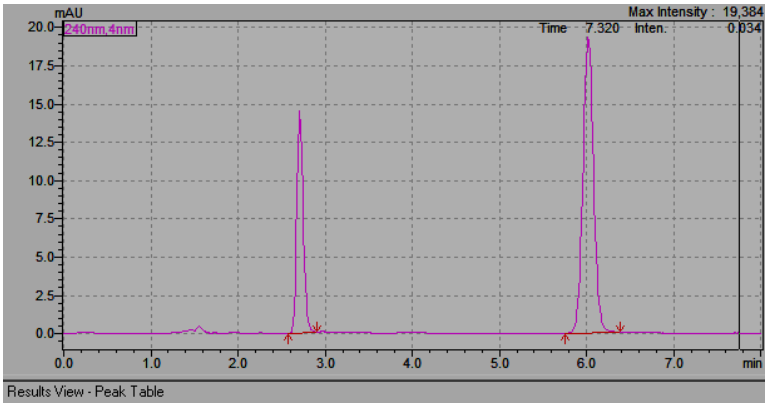


Figure 25: Chromatogram of standard solution of rosuvastatin calcium and amlodipine besylate

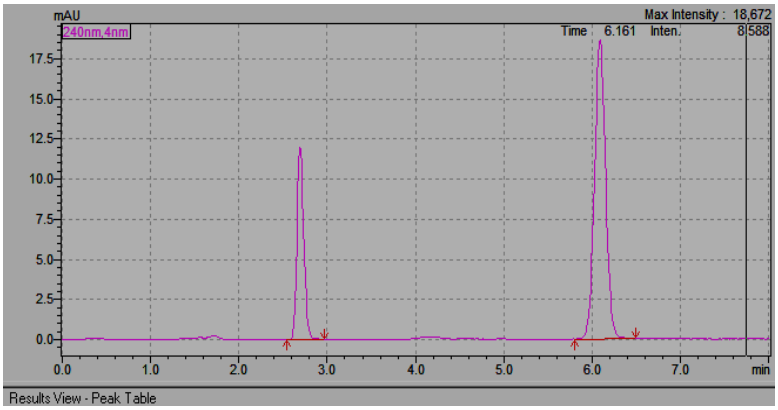


Figure 26: Chromatogram of standard solution of rosuvastatin calcium and amlodipine besylate (Interday)

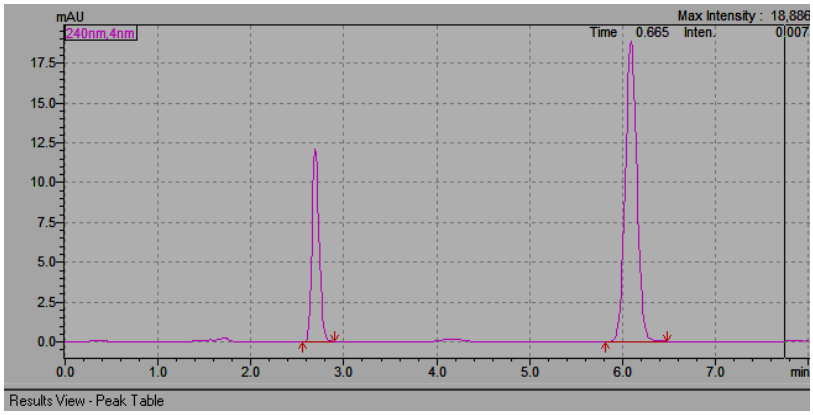


Figure 27: Chromatogram of standard solution of rosuvastatin calcium and amlodipine besylate (Intraday)

Table 13: Result of Precision study of rosuvastatin calcium and amlodipine besylate

Injected no.	Rosuvastatin				Amlodipine			
	Interday		Intraday		Interday		Intraday	
	Standard	Sample	Standard	Sample	Standard	Sample	Standard	Sample
1	161560	158933	163973	162000	64055	71861	65079	73119
2	161428	159011	164267	162122	64203	71873	65029	73112
3	161672	158852	163975	161721	64265	71518	65072	73337
4	161567	158983	164448	161825	64275	71821	65027	73121
5	161488	159269	164367	161773	64295	71847	65034	73051
6	161504	158835	165454	161530	64278	71573	65077	73031
Average	161536.5	158980.5	164414	161828.5	64228.5	71748.83	65053	73128.5
Standard deviation	83.69	157.55	546.59	209.43	90.69	159.40	25.40	109.00
%RSD	0.052	0.099	0.332	0.129	0.141	0.222	0.039	0.149

Data interpretation:

It is observed from the above tabulated data (Table.13) that the method is precise as the relative standard deviation of the sample and standard preparation of rosuvastatin and amlodipine is ≤ 2%.

3.2.5. Ruggedness

Ruggedness is a measure of reproducibility of test results under the variation in conditions normally expected from laboratory to laboratory and from analyst to analyst. To determine ruggedness of the proposed method, test sample solution was analyzed in five replicates comparing percentage relative standard deviation of the measurement of the two analysts in the same laboratory.

Table 14: Result of Ruggedness study of rosuvastatin calcium and amlodipine besylate

	Analyst-1		Analyst -2	
Injected no	Rosuvastatin Calcium (Peak Area)	Amlodipine Besylate (Peak Area)	Rosuvastatin Calcium (Peak Area)	Amlodipine Besylate (Peak Area)
1	160640	63482	160638	63472
2	160496	63561	160399	63500
3	160357	65404	160368	65358
4	160399	63498	160456	63440
5	160400	63874	160445	63456
6	160193	63496	160333	63596
Average	160414	63886	160440	63804
Standard deviation	148.5	758.4	107.5	763.5
%RSD	0.09	1.187	0.07	1.20

Data interpretation:

From the above data (Table 14) it can be concluded that, the results are within the limit. Therefore, the method is rugged.

3.2.6. Limit of Quantitation

The limit of quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be quantitated. The quantitation limit is determined by the analysis of sample with known concentration of analyte and by establishing the minimum level at which the analyte can be reliably estimated with acceptable precision, accuracy under the stated experimental

conditions. The LOQ values were determined by formulae LOQ = 10 σ/m (where, σ is the standard deviation of the responses and m is the mean of the slope of the calibration curve).

Table 15: Result of LOQ of rosuvastatin calcium and amlodipine besylate

	Signal height	Concentration (µg/ml)
Rosuvastatin calcium	399	0.22
Amlodipine besylate	401	0.095

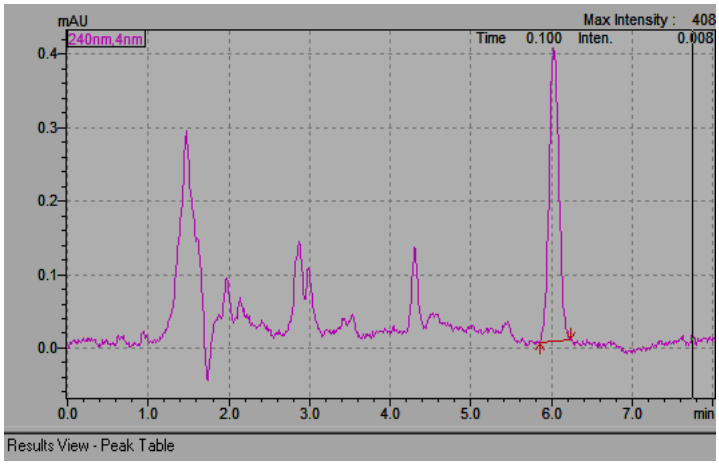


Figure 28: Chromatogram of LOQ study of rosuvastatin calcium (dilution 4)

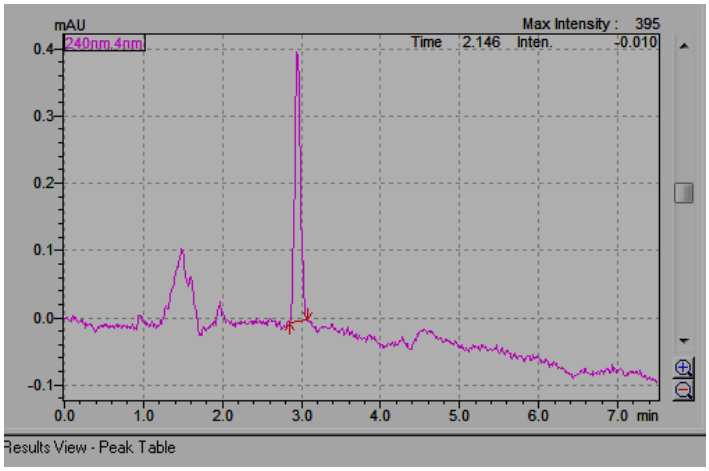


Figure 29: Chromatogram of LOQ study of amlodipine besylate (dilution 5)

- Dilution of rosuvastatin calcium:

25 mg of rosuvastatin calcium was accurately weighed into 50 ml volumetric flask. The contents were dissolved using mobile phase, sonicated and the volume was made up to 50 ml with mobile phase mixture. This is the stock solution of rosuvastatin.

Dilution 1

22 ml of the stock solution of rosuvastatin calcium was taken in 100 ml volumetric flask and volume was made up to 100 ml with mobile phase mixture. This is solution A.

Dilution 2

2 ml of the solution form solution A was taken in 10 ml volumetric flask and volume was made up to 10 ml with mobile phase mixture. This is solution B.

Dilution 3

1 ml of the solution from solution B was taken in 10 ml volumetric flask and volume was made up to 10 ml with mobile phase mixture. This is solution C.

Dilution 4

1ml of the solution from solution C was taken in 10 ml volumetric flask and volume was made up to 10 ml with mobile phase. This is solution D (figure 28).

- Dilution of Amlodipine besylate:

12.5 mg of amlodipine besylate was accurately weighed into 100 ml volumetric flask. The contents were dissolved using mobile phase, sonicated and the volume was then made up to 100 ml with mobile phase. It had been named as stock solution of amlodipine.

Dilution 1

5 ml of stock solution of amlodipine was taken in 10 ml volumetric flask and volume was made up to 10 ml with mobile phase mixture. This is solution A.

Dilution 2

1 ml solution from solution A was taken in 10 ml volumetric flask and volume was made up to 10 ml with mobile phase mixture. It was named as solution B.

Dilution 3

1 ml solution from solution B was taken in 10 ml volumetric flask and volume was made up to 10 ml with mobile phase mixture. It was named as solution C.

Dilution 4

1 ml solution from solution C was taken in 10 ml volumetric flask and volume was made up to 10 ml with mobile phase mixture. This is solution D.

Dilution 5

1 ml solution from solution D was taken in 10 ml volumetric flask and volume was made up to 10 ml with mobile phase mixture. This is solution E (Figure 29).

Data Interpretation:

The sample concentration o up to 0.095 µg/ml of rosuvastatin and 0.22 µg/ml of amlodipine can be readily quantified with the accepted accuracy (Table 15).

3.2.7. Limit of Detection

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected. The detection limit is determined by the analysis of sample with known concentration of analyte and by establishing the minimum level at which the analyte can be reliably detected. The LOD values were determined by formulae LOD = 3.3 σ/m (where, σ is the standard deviation of the responses and m is the mean of the slope of the calibration curve).

Table 16: Result of LOD of rosuvastatin calcium and amlodipine besylate

	Signal height	Concentration (µg/ml)
Rosuvastatin calcium	111	0.06
Amlodipine besylate	112	0.018

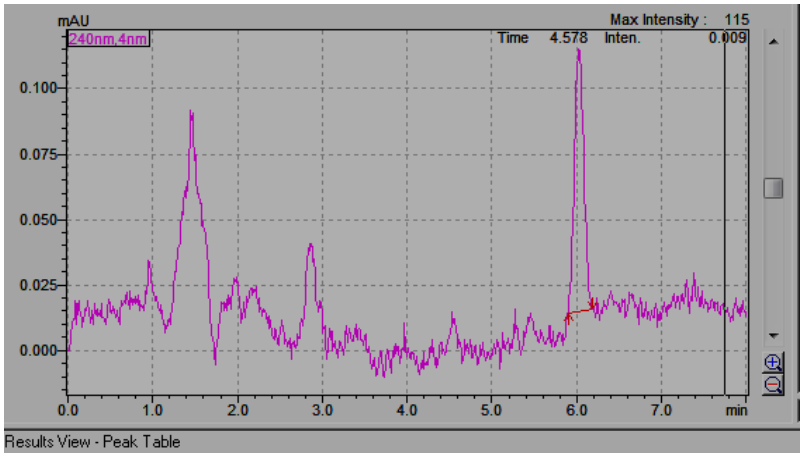


Figure 30: Chromatogram of LOD study of rosuvastatin calcium (dilution 4)

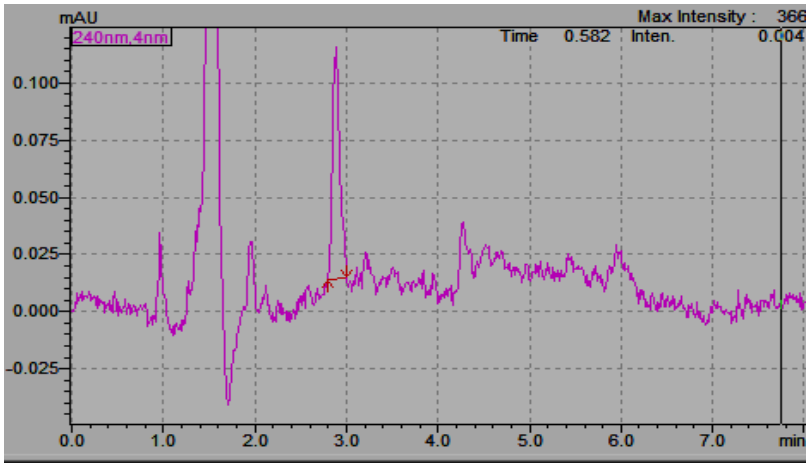


Figure 31: Chromatogram of LOD study of amlodipine besylate (dilution 5)

- Dilution of Rosuvastatin calcium:

25 mg of rosuvastatin calcium was accurately weighed in 50 ml volumetric flask. The contents were dissolved using mobile phase, sonicated and the volume was made up to 50 ml with mobile phase mixture. This is stock solution of rosuvastatin.

Dilution 1

12 ml solution from the stock solution of rosuvastatin calcium was taken in a 100 ml volumetric flask and volume was made up to 100 ml with mobile phase mixture. This is solution A.

Dilution 2

1 ml solution from solution A was taken in a 10 ml volumetric flask and volume was made up to 10 ml with mobile phase mixture. This is solution B.

Dilution 3

1 ml solution from solution B was taken in a 10 ml volumetric flask and volume was made up to 10 ml with mobile phase mixture. This is solution C.

Dilution 4

1ml solution from solution C was taken in 10 ml volumetric flask and volume was made up to 10 ml with mobile phase mixture. This is solution D (Figure 30).

- Dilution of Amlodipine besylate:

12.5 mg of amlodipine besylate was accurately weighed in 100 ml volumetric flask. The contents were dissolved using mobile phase, sonicated and the volume was then made up to 100 ml with mobile phase. This is stock solution of amlodipine

Dilution 1

5 ml solution was taken out from the stock solution of amlodipine besylate in a 10 ml volumetric flask and volume was made up to 10 ml with mobile phase mixture. This is solution A.

Dilution 2

3 ml solution from solution A was taken in a 10 ml volumetric flask and volume was made up to 10 ml with mobile phase mixture. This is solution B.

Dilution 3

1 ml solution from solution B was taken in a 10 ml volumetric flask and volume was made up to 10 ml with mobile phase. This is solution C.

Dilution 4

1 ml solution from solution C was taken in 10 ml volumetric flask and volume was made up to 10 ml with mobile phase mixture. This is solution D.

Dilution 5

1 ml solution from solution D was taken out in a 10 ml volumetric flask and volume was made up to 10 ml with mobile phase mixture. This is solution E (Figure 31).

Data Interpretation:

The sample concentration up to 0.06 µg/ml of rosuvastatin and 0.018 µg/ml of amlodipine can be readily detected with the accepted accuracy (Table 16).

3.2.8. Robustness

The robustness of an analytical procedure refers to its ability to remain unaffected by small and deliberate variations in method parameters and provides an indication of its reliability for routine analysis (Shabir). To determine robustness of the proposed method, % test sample preparations were prepared and analyzed by varying analytical parameters while keeping the other parameters unchanged such as the composition of mobile phase (±5%), flow rate (±2%), column temperature (±5ºC), wavelength (±5) (Figure 32-38).

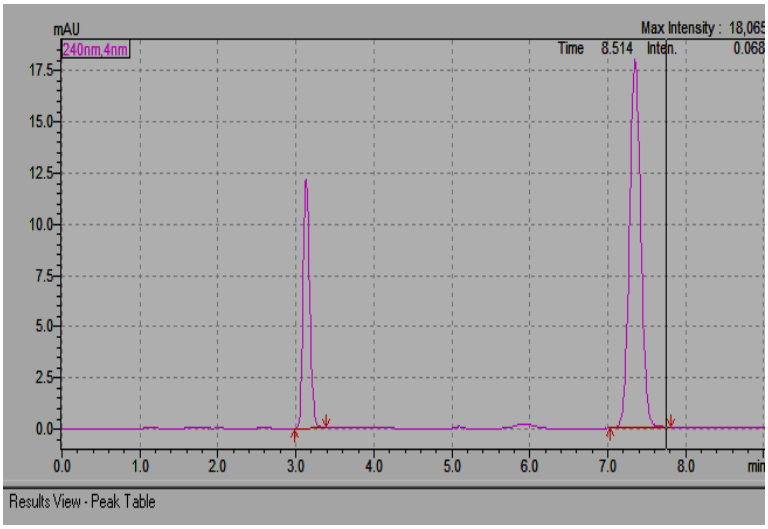


Figure 32: Chromatogram of rosuvastatin and amlodipine at a flow-rate of 1.3 ml/min

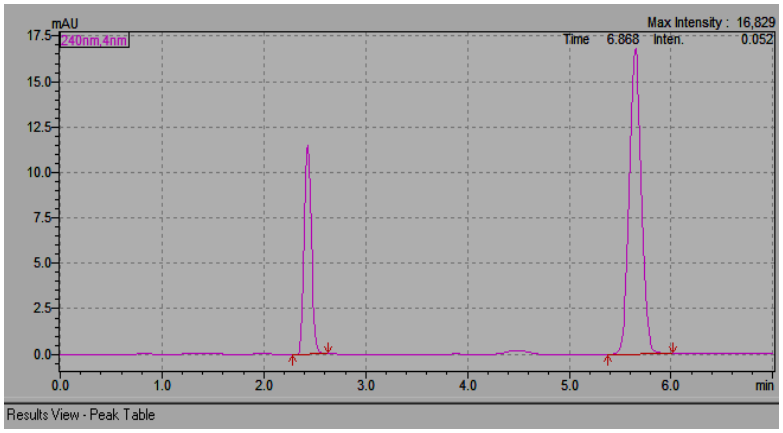


Figure 33: Chromatogram of rosuvastatin and amlodipine at a flow-rate of 1.7 ml/min

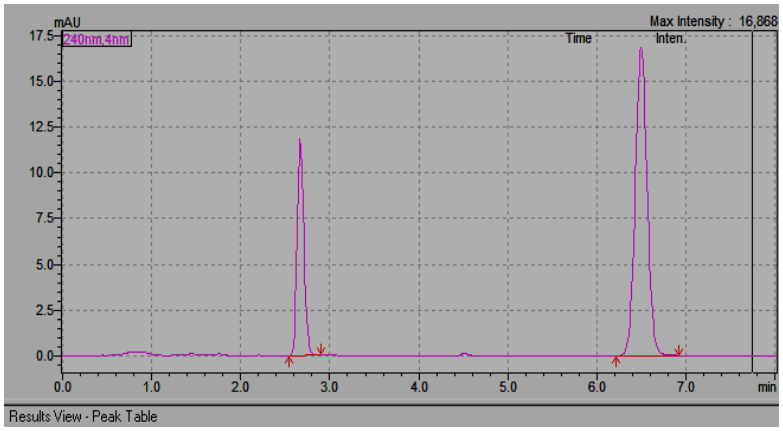


Figure 34: Chromatogram of rosuvastatin calcium and amlodipine besylate at 20°C

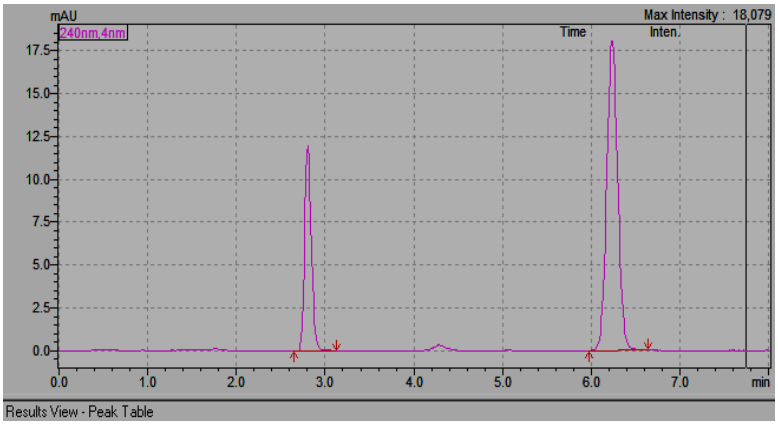


Figure 35: Chromatogram of rosuvastatin calcium and amlodipine besylate at 30°C

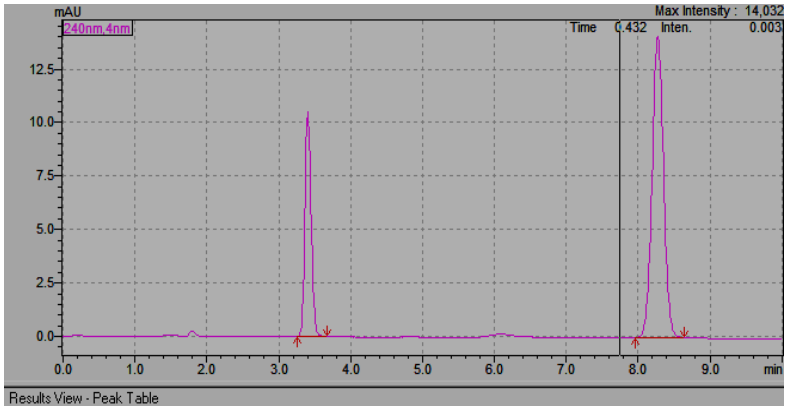


Figure 36: Chromatogram of rosuvastatin calcium and amlodipine besylate at a mobile phase ratio ACN:Buffer (42:58)

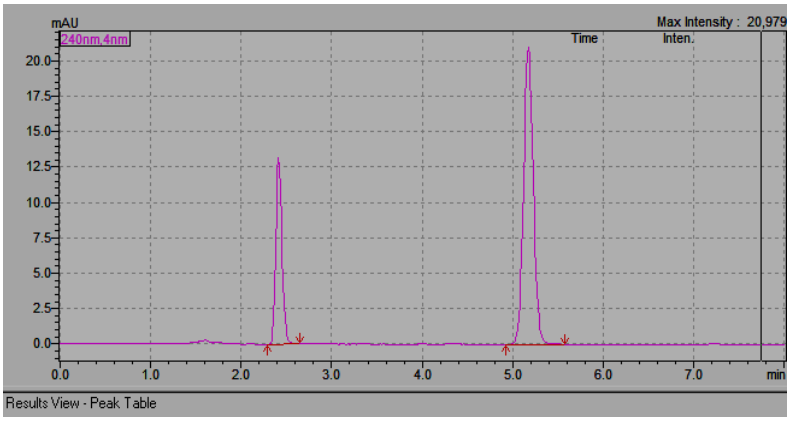
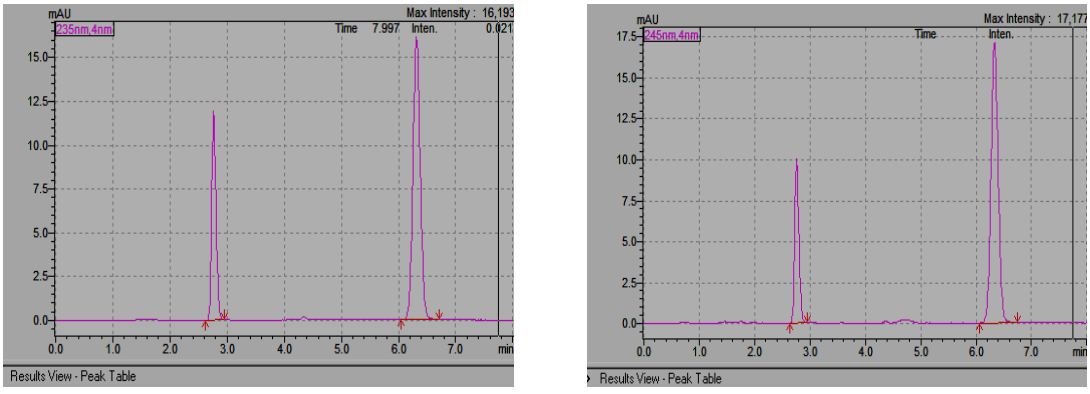


Figure 37: Chromatogram of rosuvastatin calcium and amlodipine besylate at a mobile phase ratio ACN:Buffer (48:52)



(a) (b)

Figure 38: Chromatogram of rosuvastatin and amlodipine at (a) 235 nm & (b) 245 nm

Table 17: Result of robustness study of rosuvastatin calcium

Rosuvastatin	Flow rate		Mobile phase composition`		Column temperature		Wavelength	
	1.3 ml/min	1.7 ml/min	ACN:Buffer (48:52)	ACN:Buffer (42:58)	20ºC	30ºC	235 nm	245 nm
1	72314	139539	158562	155336	157502	157417	156999	157347
2	72381	139187	157896	155503	157219	157006	157532	157432
3	72315	139253	158761	155407	157314	157315	157515	157515
4	72387	139186	158645	155371	157478	157259	157466	157966
5	72358	139180	158466	155352	157402	157245	157469	157943
6	72355	139243	158021	155273	157417	157240	157679	157529
Avg.	72352	139265	158392	155374	157389	157247	157443	157622
STD.	31.4	138	351.6	77.3	106	135.5	231.1	265.8
%RSD	0.04	0.10	0.22	0.22	0.07	0.09	0.15	0.17

Table 18: Result of robustness study of amlodipine besylate

Amlodipine	Flow rate		Mobile phase composition`		Column temperature		Wavelength	
	1.3 ml/min	1.7 ml/min	ACN:Buffer (48:52)	ACN:Buffer (42:58)	20ºC	30ºC	235 nm	245 nm
1	72314	55381	62807	62755	62952	63505	63128	63124
2	72381	55494	62623	62857	63692	63349	63043	63455
3	72356	55412	62766	62681	62789	63413	63343	63298
4	72325	55424	62759	62799	62815	63476	63233	63455
5	72366	55476	62883	62746	62833	63442	63127	63120
6	72348	55437	62767	62767	63016	63437	63175	63290.4
Avg.	72348	55437	62768	62768	63016	63437	63175	63290.4
STD.	25.1	42	84.7	58.5	342	53.8	103.5	149.0
%RSD	0.03	0.08	0.13	0.13	0.54	0.08	0.16	0.24

Data interpretation:

From the above data (Table 17 & 18) it can be concluded that, the results are within the limit. Therefore, the method is robust.

3.3. Data of in-vitro dissolution study

In the previous section of the study, the validation of the method for estimation of rosuvastatin calcium and amlodipine besylate using was done using reverse phase C-18 column (250 x 4.6 mm, 5 µm) at a wavelength of 240 nm in mobile phase composition containing phosphate buffer (pH 2.5) and acetonitrile in the ratio 55:45 % (v/v). This same method was also used for the comparative in-vitro dissolution study of formulated combination preparations of rosuvastatin & amlodipine with their separate formulation available in the market. For the estimation of the particulate release of rosuvastatin and amlodipine, separate dissolution mediums were used according to FDA dissolution specifications. For the in vitro dissolution study, three formulated

combination preparations of rosuvastatin & amlodipine were compared with the three separate market preparations of amlodipine as well as three separate market preparations of rosuvastatin.

A typical acceptance criterion for dissolution release of drugs from immediate release tablet is about 80% of label amount in 45 minutes. The *in vitro* dissolution profile of the combination formulation tablets of rosuvastatin and amlodipine was compared with that of separate commercial preparations of amlodipine and rosuvastatin alone by using the proposed HPLC method that are shown in Figures 39 & 40. Both marketed and combined formulation preparations released on an average 95% rosuvastatin within 45 min whereas on an average 90% of amlodipine was released within 45 min from both marketed and formulated preparations (tables 19 & 20). The dissolution pattern complies with the BP Guidance standards as well as with the in-house specifications (rosuvastatin calcium is an INN drug), indicating suitability of the proposed method for the dissolution study of the two drugs. The result of the chromatographic study of the marketed and combination preparation of rosuvastatin and amlodipine is shown in the following tables (Table 21-24).

Table 19: Dissolution profile of rosuvastatin calcium

Rosuvastatin calcium			
Time interval	Dissolution media	% of drug release	
		Formulated combination preparation	Market preparation
After 10 min	0.05 M sodium citrate buffer of pH 6.6	88.03	83.89
After 20 min		91.65	90.86
After 30 min		94.06	92.7
After 45 min		96.99	94.07
After 60 min		98.5	98

Table 20: Dissolution profile of amlodipine besylate

Amlodipine besylate			
Time interval	Dissolution media	% of drug release	
		Formulated combination preparation	Market preparation
After 10 min	0.01 N HCl	58.69	90.08
After 20 min		71.56	92.16
After 30 min		83.62	98
After 45 min		92.56	102
After 60 min		99.65	105

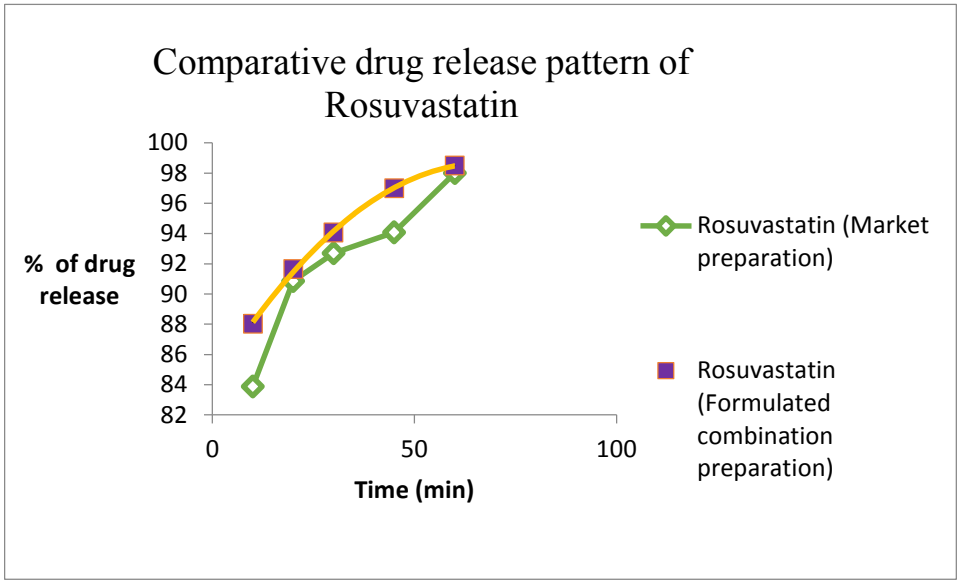


Figure 39: Drug release pattern of rosuvastatin calcium from formulated and market preparation

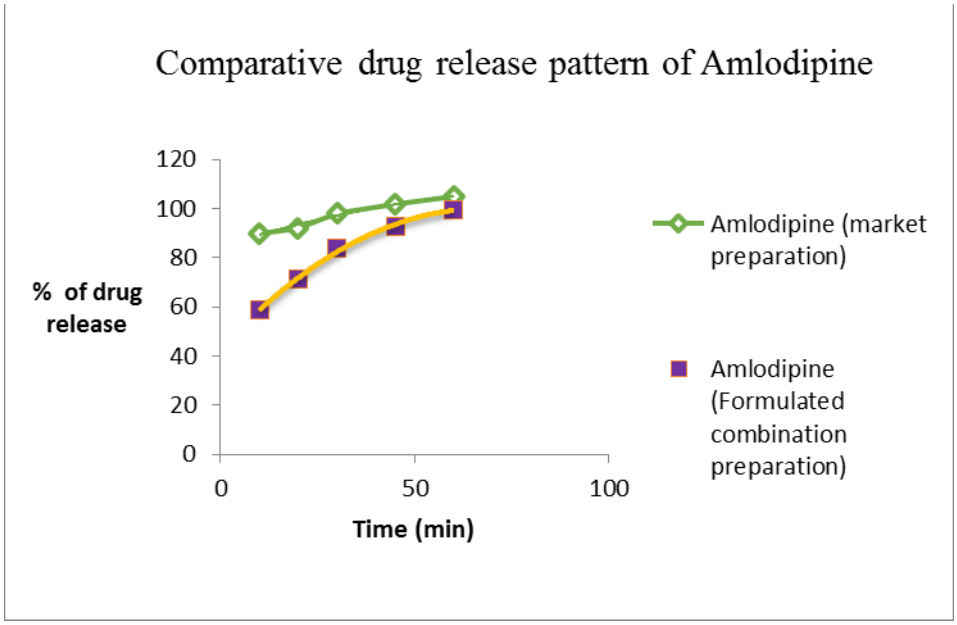


Figure 40: Drug release pattern of amlodipine besylate from formulated and market preparation

Table 21: Summary of chromatogram of rosuvastatin calcium in formulated Tablets

Rosuvastatin calcium		Retention Time	Tailing Factor	Theoretical Plate	Peak Area
Formulated Tablet 1	10 min	6.188	1.032	10791	160162
	20 min	6.186	1.037	10735	158866
	30 min	6.182	1.033	10707	161491
	45 min	6.185	1.033	10841	137665
	60 min	6.184	1.036	10730	168484
Formulated Tablet 2	10 min	6.188	1.033	10773	164960
	20 min	6.183	1.036	10756	164678
	30 min	6.182	1.033	10708	166979
	45 min	6.18	1.032	10699	177800
	60 min	6.187	1.033	10848	160015
Formulated tablet 3	10 min	6.183	1.036	10677	158950
	20 min	6.189	1.034	10749	162544
	30 min	6.187	1.035	10764	163406
	45 min	6.184	1.033	10717	168975
	60 min	6.198	1.033	10842	160153
Average		6.186	1.034	10756	162342
Standard deviation		0.004	0.00162	54.474	8467.9
Relative standard deviation (%)		0.069	0.1571	0.5065	5.21611

Table 22: Summary of chromatogram of rosuvastatin calcium in marketed preparations

Rosuvastatin calcium		Retention Time	Tailing Factor	Theoretical Plate	Peak Area
Market Rosuvastatin tablet 1	10 min	6.213	1.03	10875	152098
	20 min	6.163	1.033	10891	160000
	30 min	6.15	1.035	10637	166659
	45 min	6.158	1.034	10530	167584
	60 min	6.177	1.034	10585	169812
Market Rosuvastatin tablet 2	10 min	6.209	1.032	10961	160137
	20 min	6.144	1.034	10772	163603
	30 min	6.158	1.034	10633	166850
	45 min	6.169	1.036	10530	162337
	60 min	6.184	1.036	10481	175513
Market Rosuvastatin tablet 3	10 min	6.18	1.033	10875	174611
	20 min	6.154	1.036	10772	176374
	30 min	6.163	1.038	10594	183467
	45 min	6.178	1.031	10474	184606
	60 min	6.192	1.033	10429	189395
Average		6.173	1.034	10669	170203
Standard Deviation		0.02	0.00209	174.3	10374.7
Relative standard deviation (%)		0.332	0.2018	1.63	6.095

Table 23: Summary of chromatogram of amlodipine besylate in formulated Tablets

Amlodipine besylate		Retention time	Tailing Factor	Theoretical Plate	Peak Area
Formulated Tablet 1	10 min	2.594	1.149	6311	100279
	20 min	2.597	1.148	6328	100556
	30 min	2.59	1.162	6502	113767
	45 min	2.594	1.153	6325	115617
	60 min	2.591	1.164	6485	115118
Formulated Tablet 2	10 min	2.595	1.148	6317	101213
	20 min	2.596	1.148	6327	103111
	30 min	2.586	1.146	6309	110551
	45 min	2.603	1.153	6617	112022
	60 min	2.589	1.164	6376	115279
Formulated tablet 3	10 min	2.6	1.157	6473	105543
	20 min	2.596	1.146	6309	106963
	30 min	2.593	1.153	6557	107788
	45 min	2.591	1.162	6490	101802
	60 min	2.591	1.158	6530	105020
Average		2.594	1.154	6417	107642
Standard deviation		0.004	0.0066	108.04	5702
Relative standard deviation (%)		0.168	0.575	1.68	5.30

Table 24: Summary of chromatogram of amlodipine besylate in marketed preparations

Amlodipine		Retention time	Tailing Factor	Theoretical Plate	Peak Area
Market Amlodipine tablet 1	10 min	2.587	1.152	6589	133842
	20 min	2.588	1.153	6540	148779
	30 min	2.589	1.158	6444	151720
	45 min	2.584	1.153	6387	153340
	60 min	2.588	1.155	6390	154629
Market Amlodipine tablet 2	10 min	2.598	1.154	6440	137873
	20 min	2.587	1.155	6240	137781
	30 min	2.6	1.148	6389	143420
	45 min	2.596	1.147	6385	153770
	60 min	2.594	1.151	6354	156955
Market Amlodipine tablet 3	10 min	2.595	1.151	6339	132489
	20 min	2.59	1.158	6362	146266
	30 min	2.587	1.156	6358	152834
	45 min	2.588	1.168	6379	154580
	60 min	2.589	1.153	6362	156578
Average		2.591	1.154	6397	147657
Standard Deviation		0.005	0.0050	82.8	8497.4
Relative Standard Deviation (%)		0.182	0.4293	1.29	5.755

Chapter 4

Discussion

The compatibility study of rosuvastatin calcium and amlodipine besylate with the selected excipients came out positive which enabled us to adopt the formula to formulate the combination dosage form. In the data analysis of the compatibility study, transmittance of some selected functional groups have been observed and studied. The selection of the functional groups is basically done based upon the vulnerability of those functional groups in case of instability. For rosuvastatin, -OH group of alcoholic and carboxylic acid origin have been observed as they are susceptible to initiate any kind of chemical reaction and show possibility to form intermolecular and intramolecular –H bond. Another vulnerable group present both in rosuvastatin and amlodipine besylate is the sulfone group (S=O) which show susceptibility due to the presence of loan pair electrons of oxygen molecule. On the other hand, for amlodipine N-H group of primary amine, secondary amine, C=O group of α,β unsaturated ester have been observed and studied due to the presence of loan pair electron of nitrogen and oxygen molecule.

The transmittance of different functional groups of the pure sample of rosuvastatin and amlodipine in the IR spectrum were compared with the IR spectrum exhibiting transmittance of those same functional groups in presence of all the excipients individually. The expression pattern of different functional groups of rosuvastatin and amlodipine seemed uninterrupted in presence of excipient. In some spectrum transmittance peak of a particular functional group of the pure sample of rosuvastatin and amlodipine get merged with the common functional group present in the excipient whereas in some other spectrum the response of some particular functional groups of rosuvastatin and amlodipine get subside with the presence of the function group of the excipient. There is a presence of similar pattern of transmittance of the selected functional groups in the IR spectrum of the particular excipient and pure drug mixture which makes them identical to detect and enable to claim them to be compatible with the pure drug. In brief, all the excipients show compatibility with pure drug of rosuvastatin and amlodipine which ensues the certainty of formulating combination dosage form.

The proposed method describes a RP-HPLC procedure employing a Luna 5µ C18 column (250 mm x 4.60 mm) and a mobile phase composition containing acetonitrile and phosphate buffer in

the ratio 45:55 % (v/v). In order to develop the method with good resolutions, the changes in proportion of solvents were studied in the initial phase of the study. Acetonitrile, methanol, THF, phosphate buffer and water were tested in various ratios and compositions to get an appropriate mobile phase composition. The mixtures of acetonitrile, THF and water at various ratios were examined at first, which resulted in very good resolutions for the two pure drugs but using them for the estimation of the marketed preparations of the drug resulted in some broadening, disrupted peak for amlodipine. In addition, the method was not sensitive at all to detect rosuvastatin from the marketed formulation. The other reason for discarding the mobile phase composition containing acetonitrile, water, THF is the toxicity of THF and their detrimental effect after its disposal to the environment. Good resolutions for the two drugs were achieved with the mobile phase having a composition of acetonitrile and phosphate buffer in the ratio 45:55 % (v/v).

Retention time for both the drugs were also studied with flow rate of mobile phase at 1.3 ml/min, 1.5ml/min, 1.7 ml/min. Optimum retention time with greater resolution of separate peaks for the two drugs were obtained within eight minutes (approx.) with a flow rate of 1.5ml/min. 10 µg/ml concentration of two drug solutions were scanned in the UV range of 200 nm to 400 nm on an UV-Visible spectrophotometer. After recording the spectra of the two drugs, 240 nm was selected as suitable wavelength for estimation. Hence the method of acetonitrile and phosphate buffer in the ratio 45:55 % (v/v) with 1.5ml/min at the detection wavelength of 240 nm was selected for the simultaneous estimation of rosuvastatin and amlodipine.

Accuracy of the selected method was checked by adding known amount of pure drug to each known concentration of placebo at 3 different concentration levels. The resulting mixtures were run on HPLC by the proposed method. The result of analysis showed excellent recoveries for both the drugs ranging from 98 % to 102% for amlodipine & rosuvastatin which suggests the accuracy of the method for the simultaneous estimation of rosuvastatin and amlodipine. Precision of the method was reflected by percentage of relative standard deviation as 0.111 for rosuvastatin and 0.242 for amlodipine which was less than 2%. The limit of detection (LOD) and limit of quantification (LOQ) were determined by visual methods as suggested in ICH guidelines, which were found to be 0.095 µg/ml and 0.06 µg/ml, respectively for rosuvastatin and 0.018µg/ml and 0.22 µg/ml, respectively for amlodipine. The linearity response of the HPLC system for rosuvastatin was obtained in the range 8 - 1.2 µg/ml and in the range of 4-6 µg/ml for

amlodipine. The robustness of the proposed method was determined by varying different parameters and measuring their percentage of relative standard deviation. The percentage relative standard deviation was found to be less than 2 % for each of the parameters which are in the acceptable limit.

Moreover, to evaluate the sensitivity of the validated method, *in vitro* dissolution study was done to simultaneously estimate rosuvastatin and amlodipine from their formulated combined preparations and separate market formulation. After analyzing the result, it was observed that the concentration of the drugs has been increased which indicates that the separate dissolution media were suitable enough to conduct the dissolution study of the combined formulation. Furthermore, the relative standard deviation of the peak area of the formulated tablets and the separate market formulations were found very close and within 6%. Hence, the developed method was itself sophisticated enough to estimate simultaneously rosuvastatin calcium and amlodipine besylate from any tablet containing the two drugs.

Chapter 5

Concluding Remarks

The proposed combination formulation of rosuvastatin calcium and amlodipine besylate has shown compatibility with the chosen excipients, verified through FT-IR study. The proposed RP-HPLC method for the simultaneous estimation of rosuvastatin calcium and amlodipine besylate within 8 min (approx) with the use of mobile phase composition containing acetonitrile and phosphate buffer in the ratio 45:55 % (v/v) is simple, specific, precise, accurate, robust, and economic and validated as per the ICH guidelines and can be applied for the long term stability studies as well as for the kinetic studies of the pharmaceutical formulations. The analysis of combination tablet formulation containing two drugs gave the satisfactory results and the parameters for the two titled drugs met the criteria of ICH guidelines for method validation (Table 23). The recovery studies revealed excellent accuracy and high precision of the method. Therefore, it can be concluded that the RP-HPLC method developed in this study can be conveniently adopted for the routine quality control analysis in the combination formulations.

The present study can be conveniently applied for the routine analysis of the assay and dissolution study of the combination formulation of rosuvastatin calcium and amlodipine besylate. As the results of the proposed combination formulation show positive remarks, preparations of the combination dosage form of rosuvastatin calcium and amlodipine besylate which is not currently available in the market, can be thought of as a formulation and the method developed in this study can be reported as an analytical method validation protocol.

Table 25: Summary of the validation of assay study of Rosuvastatin calcium and Amlodipine besylate

Validation Parameter	Acceptance criteria	Results	
		Amlodipine Besylate	Rosuvastatin Calcium
System suitability	The %RSD value of peak area, tailing factor, theoretical plate, retention time for each peak of rosuvastatin calcium and amlodipine besylate should be NMT 2% for three replicate injections.	0.308	0.060
Linearity	The Correlation Co-efficient (R^2) should be NLT 0.995 for both rosuvastatin calcium and amlodipine besylate.	0.995	0.992
Accuracy	Mean % recovery at each level should be between 98% & 102% for both rosuvastatin calcium and amlodipine besylate.	Mean % recovery at each level was found to be between 98% & 102% for both rosuvastatin calcium and amlodipine besylate.	
Precision	The % RSD value of the peak area of rosuvastatin calcium and amlodipine besylate obtained from six replicate injections should be NMT 2%	The RSD value of Peak area of rosuvastatin calcium and amlodipine besylate was found within the limit.	
Ruggedness	The %RSD value of the peak area of rosuvastatin calcium and amlodipine besylate obtained from six replicate injections (done by two different analyst) should be NMT 2%.	The RSD value of Peak area of rosuvastatin calcium and amlodipine besylate was found within the limit.	
Robustness	The RSD value of the peak area of rosuvastatin calcium and amlodipine besylate obtained from changing different parameter (like – mobile phase ratio, wavelength, temperature flow rate) should be within 2 %.	The RSD value of Peak area of rosuvastatin and amlodipine was found within the limit.	

Appendix

Appendix 1

Statins

Statins, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, have shown revolution in the treatment of hypercholesterolemia. They are more effective than other lipid-lowering medications in reducing low-density lipoprotein cholesterol (LDL-C) and total cholesterol (total-C) concentrations and are the first choice of drug therapy when this is the primary goal of treatment. It competitively blocks HMG-CoA reductase enzyme with respect to the binding of the substrate, HMG-CoA since the chemical structure of statin possesses an analogue of the target enzyme substrate, HMG-CoA. In addition to that, a complex hydrophobic ring structure is covalently linked to the substrate analogue which is involved in binding of the statin to the reductase enzyme.

The liver is the primary site of action at which the statin inhibits the biosynthesis of cholesterol. Statins mimic the natural substrate molecule, HMG-CoA and act by competitively blocking the HMG-CoA reductase enzyme, which catalyzes the rate-limiting step in de novo cholesterol synthesis (Maron et al., 2000). This competition slows the rate of mevalonate production, the next molecule in the serial steps to produce cholesterol (Statins: Controlling Cholesterol). Hence, in the presence of statins, the precursor HMG-CoA is not efficiently processed forward to produce mevalonate, blocking the pathway (Statins: Controlling Cholesterol). Liver cells sense the reduced levels of liver cholesterol production with statin use and try to compensate by synthesizing more LDL receptors on the cell surface to increase cholesterol uptake from serum Statins: Controlling Cholesterol). The LDL and VLDL particles bind and are internalized into liver cells, where the cholesterol component is processed into bile salts which clear LDL and LDL precursors from the circulation. Plasma levels of low density lipoproteins (LDLs) are positively correlated with the incidence of coronary artery disease (CAD). Thus statin therapy significantly reduces lipid levels and diminishes the incidence of coronary events in individuals with stroke and ischemic heart disease.

Inhibition of mevalonate systhesis inhibits the synthesis of isoprenoid geranylgeranlpyrp phosphate (GGPP) upstream of cholesterol (Thimmaraju et al., 2013). Mevastatin was the first

HMG-CoA reductase inhibitor which was isolated from Penicillum citrinum. Other statins such as simvastatin, lovastatin and pravastatin are fungal derivatives, while atorvastatin, cerivastatin, fluvastatin, pitavastatin and rosuvastatin are fully synthetic compounds. As of now, some of the commercially marketed statins are atorvastatin (Lipitor), fluvastatin (Lescol), lovastatin (Mevacor), simvastatin (Zocor), pitavastatin (Livalo) and rosuvastatin (Creastor) which varies in their lipid lowering capacity. Of the statins currently available, rosuvastatin is the most effective in lowering LDL-C, with reductions upto 63% reported in the daily dose of 40 mg (Thimmaraju et al., 2013). Besides, several combined preparations of statin, with other cholesterol lowering drug such as ezetimibe/simvastatin, or with Ca-channel blocker atorvastatin/amlodipine, has benefited the patients in achieving recommended lipoprotein level, leading to a decrease in the incidence of cardiovascular disease.

Hepatoselectivity of statins is determined in large part by their hydrophilic properties. Hydrophobic statins tend to have higher exposure in non-hepatic tissues, resulting in unwanted side effects at other tissues. On the other hand, the hydrophilic statins are more liver specific and give them better potency. Atorvastatin, Fluvastatin, Lovastatin and Simvastatin are relatively lipophilic compounds, while Pravastatin and Rosuvastatin are more hydrophilic as a result of a polar hydroxyl group and methane sulphonamide group, respectively (Srinivasa et al., 2011). Of the marketed product, cerivastatin (Baycol) was the most lipophilic and able to diffuse into many cell types and exhibiting the most serious adverse effects before it was withdrawn in 2001 (Statins: Controlling Cholesterol). In addition, comparison of the six statin–enzyme complexes revealed subtle differences in their modes of binding. An additional hydrogen bond was demonstrated in the atorvastatin– and rosuvastatin–enzyme complexes along with a polar interaction unique to Rosuvastatin, such that Rosuvastatin has the most binding interactions with HMG-CoA reductase of all the statins (Srinivasa et al., 2011).

Appendix 2

Calcium Channel Blocker

The calcium channel blocking drugs (CCBs) are a heterogeneous group of compounds that are classified according to chemical structure: diphenylalkylamines (verapamil), benzothiazepines (diltiazem), dihydropyridines (nifedipine, amlodipine, felodipine, nimodipine), and diphenylpiperazines (flunarizine) (Drug reference. 2003). They are among the most widely used

drugs in cardiovascular medicine with roles not only in hypertension but also in angina and tachyarrhythmia. The three classes of CCBs differ not only in their basic chemical structure, but also in their relative selectivity toward cardiac versus vascular L-type calcium channels. Dihydropyridine, one of the chemical classes of Ca channel blocker**,** has minimal effect on cardiac conduction or heart rate, while they have potent actions as vasodilators because of their high selectivity to vascular smooth muscle. Although *in vitro* the dihydropyridines can depress myocardial contractility because of their reflex- mediated sympathetic stimulation on both heart rate and contractility. This cardiac stimulation has been associated with the precipitation or worsening of angina or even the occurrence of myocardial infarction or sudden death. Reflex-mediated cardiac stimulation is less likely with the longer-acting and slow-release preparations because their slower onset of effect allows baroreflex resetting.

Amlodipine, considered as a third generation member of dihydropyridine class of calcium antagonists with a long duration of action, are primarily used to treat hypertension. It inhibits transmembrane influx of extracellular calcium ions across the membranes of myocardial cells and vascular smooth muscle cells by selectively blocking voltage-gated L- type calcium channels, without changing serum calcium concentrations. When inward calcium flux is inhibited, vascular smooth muscle cells relax, resulting in vasodilation of coronary artery and arteriole smooth muscle. Vasodilation decreases total peripheral resistance which decreases cardiac output. Since blood pressure is determined by cardiac output and peripheral resistance, blood pressure drops. Thus lowering of blood pressure will substantially reduce the risk of fatal and nonfatal cardiovascular events, primarily strokes and myocardial infarctions. It is also indicated for the symptomatic treatment of chronic stable angina because of the long duration of action. As an antianginal agent, it acts as a dilator of peripheral arteries and arterioles which subsequently reduces the total peripheral resistance and, therefore, reduces the workload of the heart (after load). The unloading of the heart thereby decrease ischemia and relieve effort angina by reducing myocardial energy oxygen consumption and oxygen requirements.

CCBs are a class of drugs that should not be prescribed as initial or first line treatment in people with high blood pressure who have no other form of heart disease (Kabir et al., 2014). Amlodipine, a third generation member of dihydropyridine class of CCBs, has chosen for a combination with statins which together provide dose related reduction in systolic blood pressure, diastolic blood pressure and LDL-C in patients with co-morbid hypertension and

dyslipidemia. Amlodipine is the most reasonable choice among all the other classes of available antihypertensive drug such as beta blocker, ACE inhibitor, diuretics and the other calcium channel blockers. Compared with other CCBs, amlodipine has greater membrane affinity, owing to its positive charge and strong lipophilicity which increases its oral bioavailability. The longer duration of elimination half life prolongs the duration of action which decreases the dosing frequency and maintains a uniform concentration of the drug. Amlodipine also has antioxidant effects, independent of calcium channel modulation, and a vasodilatory effect via the inhibition of nitric oxide release, which inhibits platelet aggregation. These pleiotropic effects of amlodipine suggest that it is more cardioprotective than other non-CCB-based treatments (Park, 2014). Amlodipine does not appear to increase neurohormonal activity (epinephrine, renin, aldosterone, atrial natriuretic peptide) which also suggests that amlodipine may be safer than other CCBs in patients with left ventricular dysfunction or heart failure (Drug reference. 2003). The side effects of amlodipine is also less than the other calcium channel blockers which further can be minimized by keeping the dose small.

Appendix 3

Rosuvastatin calcium

Rosuvastatin calcium, chemically described as *bis [(E)-7 [4-(4-fluorophenyl)-6 isopropyl-2[methyl (methyl-sulphonyl) amino] pyrimidin-5-yl] (3R, 5S) -3,5-dihydroxyhept-6-enoic acid]* is a calcium salt of rosuvastatin. It is a member of statin used in the treatment of hyper-cholesterolemia and dyslipidemia by selective and competitive inhibition of 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme that converts HMGCoA to mevalonate a precursor of cholesterol and thereby checks the synthesis of cholesterol.

Formula: $(C_{22}H_{27}FN_3O_6S)_2Ca$

Molecular weight: 1001.14

Category: Lipid lowering agent

Pharmacologic class: Synthetic statin

Structure:

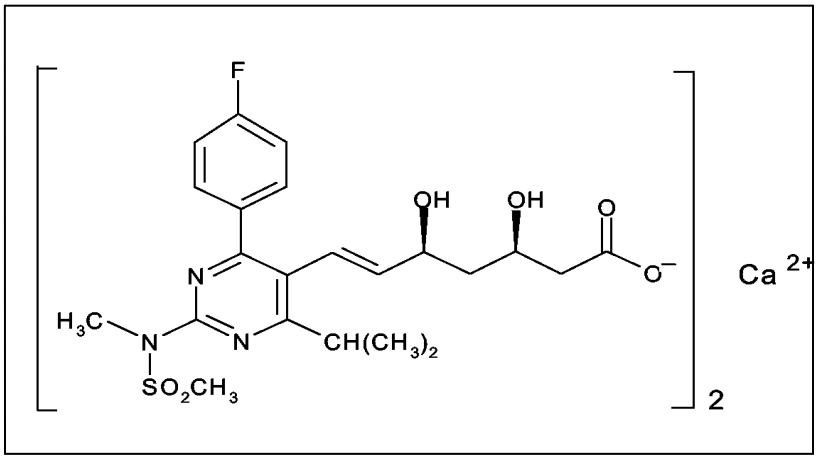


Figure 41: Structure of rosuvastatin calcium

Physicochemical properties: Rosuvastatin calcium is a hydrophilic white amorphous powder with a partition coefficient (octanol/water) of 0.13 at pH of 7.0.

Solubility: It is sparingly soluble in water and methanol, and slightly soluble in ethanol.

Mechanism of action:

Rosuvastatin is a competitive inhibitor of HMG-CoA reductase. HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonate, an early rate-limiting step in cholesterol biosynthesis. It acts primarily in the liver. Decreased hepatic cholesterol concentrations stimulate increased hepatic uptake of LDL. Again, it increases the number of hepatic LDL (Low Density Lipoprotein) receptors on the cell-surface to enhance uptake and catabolism of LDL (Anuradha et al., 2010). Moreover, it inhibits hepatic synthesis of very low density lipoprotein (VLDL), thus depletion in plasma LDL and VLDL level.

Available formulation:

Rosuvastatin is available as single pill formulations to impart effective therapeutic effect.

Dosing Information:

Rosuvastatin calcium is supplied in tablets in amounts equivalent to 5 mg, 10 mg, 20 mg, and 40 mg of Rosuvastatin. In the treatment of hypercholesterolemia, the usual recommended starting dosage is 10 mg once a day. An initial dosage of 5 mg once a day should be considered for patients requiring less aggressive LDL-C reductions or who have predisposing factors for myopathy since higher doses of statins are associated with a greater incidence of myopathy (Rosuvastatin, Crestor). For patients with marked hypercholesterolemia and aggressive lipid targets, a starting dosage of 20 mg once a day may be considered (Rosuvastatin, Crestor). A dosage of 40 mg once a day should be reserved for those patients who have not achieved the LDL-C goal at a dosage of 20 mg (Rosuvastatin, Crestor).

Pharmacokinetics:

a) **Absorption:**

- Bioavailability- 20%(first pass metabolism)
- Peak plasma conc.: 3-5 hours after oral dosing.

b) Distribution:

- Volume of Distribution: 134L
- Plasma protein binding: 88% bound to plasma proteins (mostly albumin). Binding is reversible and independent of plasma concentrations.

c) Metabolism:

Cytochrome P450 (CYP) 2C9 is primarily responsible for the formation of rosuvastatin's major metabolite, N-desmethylrosuvastatin which has approximately 50% of the pharmacological activity of its parent compound in vitro. Only ~10% is excreted as metabolite.

d) **Elimination**:

Rosuvastatin and its metabolites are primarily excreted in the feces (90%).

Appendix 4

Amlodipine Besylate

Amlodipine besylate, chemically described as *3-ethyl-5-methyl(±)-2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5 pyridinedicarboxylate, monobenzenesulphonate*, is the besylate salt of amlodipine, a long-acting dihydropyridine class of calcium channel blocker,

approved for treating hypertension and both vasospastic and chronic, stable angina (Blank, 2005).

Molecular weight: 567.05

Formula: $C_{20}H_{25}ClN_2O_5.C_6H_6O_3S$

Structure:

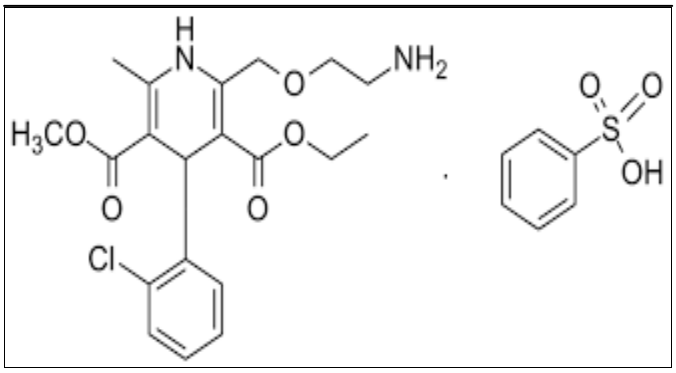


Figure 42: Structure of amlodipine Besylate

Category: Anti hypertensive agent

Pharmacologic class: Calcium L-channel antagonist.

Physical properties: Amlodipine besylate is a light sensitive white to almost white crystalline powder.

Solubility: It is freely soluble in methanol; sparingly soluble in ethanol and slightly soluble in 2-prpanol and water.

pKa: 9

Mechanism of action:

As the dihydropyridine class of drugs is more selective for vascular sites than for myocardial sites, thus, amlodipine inhibits the transmembrane influx of Ca^{2+} ion across the membranes by selectively blocking voltage-gated L- type calcium channels, without changing serum calcium concentrations. Since the influx of calcium ion through calcium channel is important for muscle contraction, by blocking calcium transport, it relaxes the muscles lining the arteries and lower blood pressure. It also expands coronary arteries which increases the flow of blood to the heart

and prevent heart pain (angina) resulting from reduced flow of blood to the heart caused by coronary artery spasm (contraction).

Available Formulation:

Amlodipine is available both as single pill or combination formulations to impart effective antihypertensive effect.

Dosage and Administration: Dosage should be individualized depending on patient's tolerance and responsiveness.

a) For both hypertension and angina, the recommended initial dose is 5 mg once daily. If necessary, dose can be increased after 1 to 2 weeks to a maximum dose of 10 mg once daily.
b) Geriatrics or Patients with Impaired Renal Function: The recommended initial dose in patients over 65 years of age or patients with impaired renal function is 5 mg once daily. If required, increasing in the dose should be done gradually.
c) Patients with Impaired Hepatic Function: Dosage requirements have not been established in patients with impaired hepatic function. When amlodipine is used in these patients, the dosage should be carefully and gradually adjusted depending on patient's tolerance and response. A lower starting dose of 2.5 mg once daily should be considered.

Pharmacokinetics:

- **Absorption:**

After oral administration of therapeutic doses of amlodipine, absorption occurs gradually from the gastrointestinal tract with peak plasma concentration reached between 6 and 9 hours. Bioavailability has been estimated 60 to 65%. The bioavailability of amlodipine is not altered by the presence of food.

- **Distribution:**
    a) Volume of distribution-21 L per kg.
    b) Protein binding: Very high (> 95 %).
    c) Duration of action: 24 hours.

- **Biotransformation:**

Amlodipine is metabolized through the cytochrome P450 system, mainly via CYP 3A4 isoenzyme. It is extensively (about 90%) converted to inactive metabolites (via hepatic metabolism) with 10% of the parent compound**.**

- **Elimination:**

Elimination half life is a mean of 35 hours in healthy volunteers. It may get prolonged to a mean of 48 hours in hypertensive patients, 65 hours in the elderly, and 60 hours in patients with hepatic function impairment.

a) Renal—59 to 62% (about 5% as unchanged amlodipine).
b) Biliary/fecal—20 to 25%.
c) In dialysis—Amlodipine is not removed by hemodialysis.

Appendix-5

FT-IR

FT-IR or Fourier Transform Infrared spectroscopy is the study of the interaction of electromagnetic radiation from the IR region of the EM spectrum (4000-400) cm^{-1} with a molecule through which IR radiation is passed. The nature of interaction depends upon the functional groups present into the substance. When IR radiation passed through a sample (solid, liquid or gas), certain frequencies of the radiation are absorbed by the atoms of the substance leading to molecular vibration. The frequencies of absorbed radiation are unique for each atom or group of atom, which provide the characteristics of bonds associated with a substance. The resulting spectrum represents the molecular absorption and transmission, which can be divided into two approximate regions:

- Functional group region (4000-1500 cm^{-1}), valuable information are obtained from this region to interpret any spectrum.
- Fingerprint region (<1500 cm^{-1}), usually consists of a very complicated series of absorption that are characteristic for a particular compound. Like a fingerprint no two unique molecular structures produce the same infrared spectrum.

Infrared spectroscopy has been a useful technique for the analysis of materials in the laboratory for over seventy years. An infrared spectrum represents a fingerprint of a sample with absorption peaks which correspond to the frequencies of vibrations between the bonds of the atoms making up the material. As each different material is a unique combination of atoms, no two compounds produce the exact same infrared spectrum. Therefore, infrared spectroscopy can result in a positive identification (qualitative analysis) of every different kind of material. In addition, the size of the peaks in the spectrum is a direct indication of the amount of material present. With modern software algorithms, infrared spectroscopy is an excellent tool for quantitative analysis, making it useful for several types of analysis:

a) A qualitative fingerprinting check for the identity of raw materials used in manufacturer and for identifying drug.
b) Used in synthetic chemistry as a preliminary check for presence or absence of functional group.
c) Can be used to characterize sample
d) Used as a fingerprint test for film, coating and packing plastics
e) Can be used to detect polymorphs of drug (polymorph are different crystal form of a chemical compounds that have different physical properties)
f) To detect the stability of any substance in presence of another substance

The original instruments of IR previously were of dispersive type. This type of instrument separates the individual frequencies emitted from the infrared source by the use of prism or grating and plots a spectrum of intensity versus frequency. The detector measures the amount of energy at each frequency which has passed through the sample. A method for measuring all of the infrared frequencies simultaneously, rather than individually, was needed. In respect to sort out the problem an optical device was developed named “interferometer”. This produces a unique type of signal which has all of the infrared frequencies “encoded” into it which can be measured very quickly, usually on the order of one second or so. Thus, the time element per sample is reduced to a matter of a few seconds rather than several minutes.

Most interferometers employ a beam splitter which takes the incoming infrared beam and divides it into two optical beams. The two beams reflect off of their respective mirrors and are recombined when they meet back at the beam splitter. Because the path that one beam travels is a

fixed length and the other is constantly changing as its mirror moves, the signal which exits the interferometer is the result of these two beams "interfering" with each other (Thermo Nicolet Cooperation, 2001). The resulting signal is called an interferogram which has the unique property that every data point (a function of the moving mirror position) which makes up the signal has information about every infrared frequency which comes from the source (Thermo Nicolet Cooperation, 2001). As a frequency spectrum (a plot of the intensity at each individual frequency) is required in order to make identification, the measured interferogram signal cannot be interpreted directly. A means of "decoding" the individual frequencies is required which can be accomplished via a well-known mathematical technique called the Fourier transformation. This transformation is performed by the computer which then presents the user with the desired spectral information for analysis. In brief, the fourier transform infrared is preferred over the dispersive method because of its speed, sensitivity in measurement and non destructive technique.

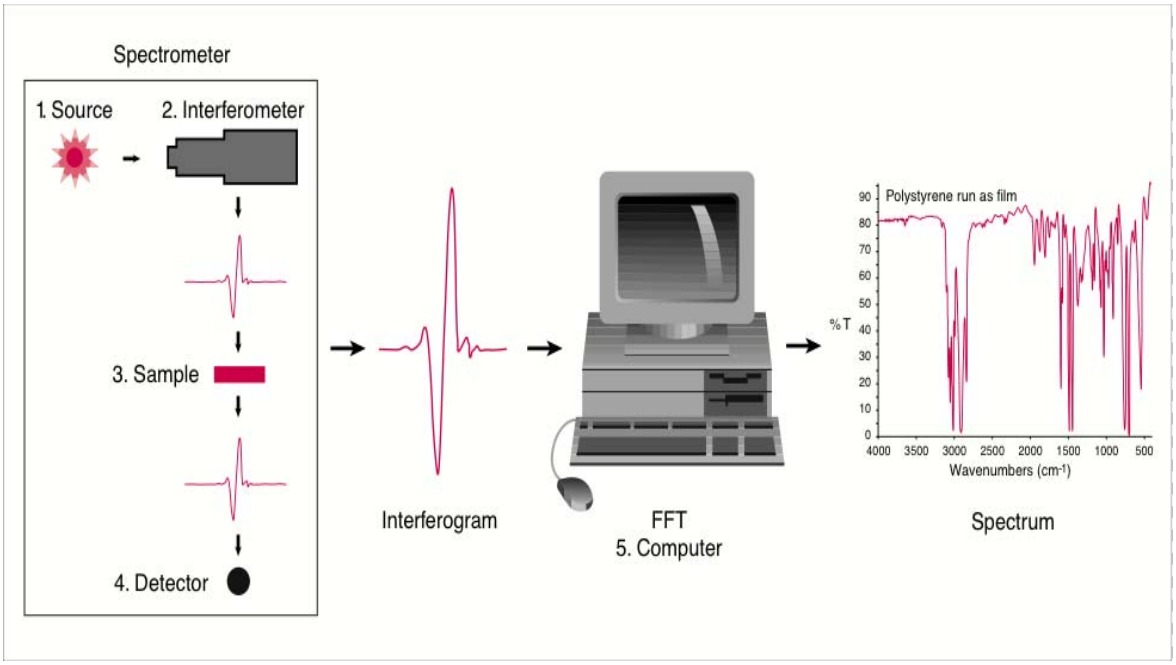


Figure 43: Instrumentation of FT-IR

Appendix 6

Reverse Phase – HPLC

Reversed-phase high-performance liquid chromatography (RP-HPLC) involves the separation of molecules on the basis of hydrophobicity. The separation depends on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase. Decreasing the mobile phase polarity by adding more organic solvent reduces the hydrophobic interaction between the solute and the solid support resulting in de-sorption. The more hydrophobic the molecule the more time it will spend on the solid support and the higher the concentration of organic solvent that is required to promote de-sorption.

In the 1970s, most liquid chromatography was performed using a solid support stationary phase containing unmodified silica or alumina resins. This method is now called "normal phase chromatography". In normal phase method, the stationary phase is hydrophilic and the hydrophilic molecules in the mobile phase will tend to adsorb to the surface on the inside and outside of hydrophilic particle. The introduction of a technique using alkyl chains covalently bonded to the solid support created a hydrophobic stationary phase, which has a stronger affinity for hydrophobic compounds, which is now known as reverse phase HPLC.

The most popular column used for reverse phase liquid chromatography are octadecyl carbon chain (C18)-bonded silica, C8-bonded silica, pure silica, cyano-bonded silica and phenyl-bonded silica and for mobile phase mixtures of water or aqueous buffers and organic solvents are usually used to elute analytes from a reversed-phase column. The solvents must be miscible with water, and the most common organic solvents used are acetonitrile, methanol, and tetrahydrofuran (THF). Other solvents can be used such as ethanol or 2-propanol (isopropyl alcohol).

Appendix-7

Dissolution

Dissolution test is required to evaluate the release of drug from a pharmaceutical dosage form as a predictor of the in vivo performance of a drug product. A dissolution test is a simple concept, where a tablet or capsule is placed into a known volume of media and as it dissolves the resulting solution is sampled over time, and assayed (often by HPLC or by spectrophotometry) for the level of active pharmaceutical ingredient (API) present. Media volumes are typically kept in the

range of 500-1000 ml, with 900 ml the most common volume. Media deaeration is usually required which can be accomplished by heating or filtering the medium or placing it under vacuum for short period of time. When developing dissolution procedure, one general goal is to have "sink" conditions. Sink conditions are defined as the volume of medium that is at least three times that required in order to form a saturated solution of drug substance (Vaghela et al., 2011). The choice of apparatus is also a matter of consideration during the method development which is based on the dosage form performance in the *in vitro* test system (Table 2) (Vaghela et al., 2011).

Table 26: USP Apparatus and Agitation Criteria

USP apparatus	Description	Rotation speed	Dosage form
I	Basket	50-120 rpm	Immediate release Delayed release Extended release
II	Paddle	25-50 rpm	Immediate release Delayed release Extended release
III	Reciprocating cylinder	6-35 rpm	Immediate release Extended release
IV	Flow through cell	25-50 rpm	Extended release, poorly soluble API
V	Paddle over disk	N/A	Transdermal
VI	Cylinder	N/A	Transdermal

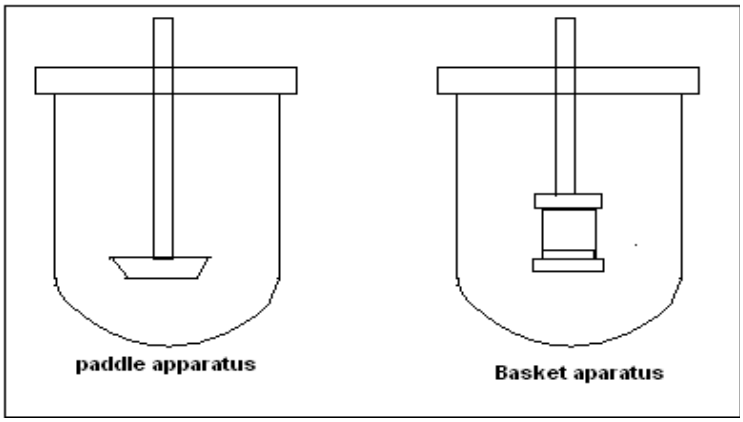


Figure 44: Dissolution Apparatus I and II

Dissolution is evaluated by measuring rate release profile or the amount dissolved over time. So, duration is another important criteria need to be considered during the method development of dissolution. For immediate release dosage forms, the procedure duration is usually 30 to 60 minutes and in most cases, single time point specification is adequate (Vaghela et al., 2011). On the other hand, for extended release dosage forms, at least three test time points are typically chosen to characterize the in vitro drug release profile (Vaghela et al., 2011). At last, for analyzing the dissolution test samples, spectrophotometric (UV) determinations and HPLC are most commonly used. When a method is developed for particular testing of any specific dosage form, that method needs to be validated for the consistency of the results for further conductance of the process.

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