1.1 Introduction

Hypertension had been recognized as an important risk factor for cardiovascular disease and is a leading risk factor for mortality (Zati et al., 2002). In the present time the various diuretic drugs that are being used in various combinations and formulations, since the target blood pressure level have been obtained by mono therapy, can be challenging, especially for patients who are suffering from other diseases. Meanwhile it is demonstrated that a majority of hypertensive patients needs two or more antihypertensive drugs to lower their blood pressure effectively. Consequently, fixed-dose, in which several active agents are combined in single pharmaceutical formulation, appears to be a novel and underlying power in overcoming the cardiovascular disease. Antihypertensive drugs, that contain more than one active ingredient, can be used as a single daily pill that shows large effect in preventing cardiovascular disease with minimal adverse effects particularly in high-risk patients who need strict blood pressure control. Multiple studies have looked into the safety and beneficial effects of initial combination therapy in patients with hypertension comparing them with those of mono therapy drugs (Wellington et al. 2002; Maurizio et al. 2008; Roberto et al. 2008; Sanjay et al. 2010; Elizabeth et al. 2011; Steven et al. 2012; Dingliang et al. 2012; Ayesha et al. 2013; Salahuddin 2013; Masatoet al. 2013; Shankar; 2014; Xinhuan et al. 2014; Soon et al. 2014).

1.1.1 Hydrochlorothiazide and Valsartan

i. Hydrochlorothiazide

The molecular formula for hydrochlorothiazide is ($C_7H_8ClN_3O_4S_2$), IUPAC name is (6-Chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulphonamide 1,1-dioxide) and the chemical structure is shown in Figure 1.1.

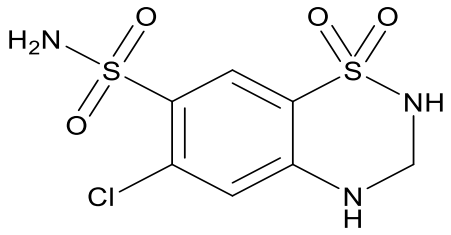

Figure 1.1 Hydrochlorothiazide structure (USP 2016)

Hydrochlorothiazide is a diuretic drug that increases the excretion of sodium, chloride and water, by inhibiting sodium ion transport in renal tubes, potassium and bicarbonate, but decreases the urinary excretion of calcium and uric acid. Hydrochlorothiazide may be used to reduce hypercalciuria. By increasing the sodium load at the distal renal tubs, hydrochlorothiazide indirectly increases potassium excretion via the sodium-potassium exchange mechanism. The efficacy of hydrochlorothiazide is not affected by the acid-base balance of the patient. It lowers blood pressure by decreasing cardiac output, reducing plasma and extracellular fluid volume. Cardiac output eventually returns to normal, plasma and extracellular fluid values return to slightly less than normal, but peripheral vascular resistance is reduced, resulting in lower blood pressure. These diuretics also decrease the filtration rate, which contributes to the drug's lower efficacy in patients with renal impairment. Hydrochlorothiazide was approved by the FDA in 1959 and it had been recommended as preferred initial therapy in patients with systemic hypertension(Welling et al. 1986 Liu et al. 2007; Ke et al. 2008; Sha et al. 2012).

ii. Valsartan

The molecular formula for valsartan is ($C_{24}H_{29}N_5O_3$), IUPAC name is (N-((2-(2H-tetrazol-5-yl)-[1,1-biphenyl]-4-yl)methyl)-N-pentanoyl-L-valine), and the chemical structure is shown in Figure 1.2.

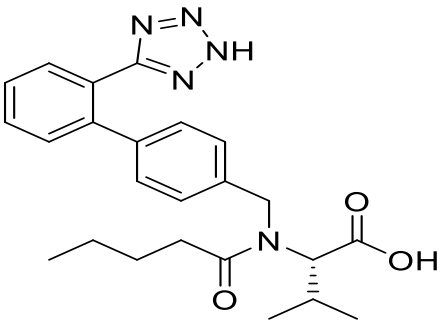

Figure 1.2 Valsartan structure. (USP 2016)

Valsartan is used to treat hypertension in adults and children up to six years old. It is effective for hypertension, either alone or in combination with other antihypertensive agents. Greater antihypertensive efficacy is achieved by adding a small dose of a diuretic to valsartan. Modest reduction in blood pressure is achieved by increasing the dose of valsartan over the range of 80—320 mg. Limited data showed that valsartan also reduces protein uria in patients with hypertension and renal disease. In addition to antihypertensive actions, it is effective in the treatment of heart failure(Pitt et al. 1997). Short-term themodynamic benefits of valsartan have been documented in patients with heart failure; it improves clinical signs in patients with heart failure(Cohn et al. 2001). There are two types of angiotensin II receptors, type (i) and type (ii). Valsartan has about a 20,000-fold greater affinity for type (i) more than for type (ii); type (ii) is not known to mediate cardiovascular homeostasis. Valsartan selectively blocks type (i) receptor in tissues such as vascular smooth muscle and the adrenal gland, it blocks the vasoconstrictor and aldosterone effects; thus by blocking the effects of type (i), it decreases systemic vascular resistance without a marked change in heart rate. Valsartan has no effect on serum uric acid. FDA approved valsartan for use in heart failure patients (August et al. 2002; Pfeiffer et al. 2002; Mehtap et al. 2007; Nadeem et al. 2011; Jin et al. 2012).

1.1.2 Amlodipine besylate and losartan potassium

i. Amlodipine besylate

The molecular formula for amlodipine besylate is ($C_{20}H_{25}ClN_2O_8S$); IUPAC name is ((RS)-3-ethyl 5-methyl 2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate) and the chemical structure is shown in Figure 1.3.

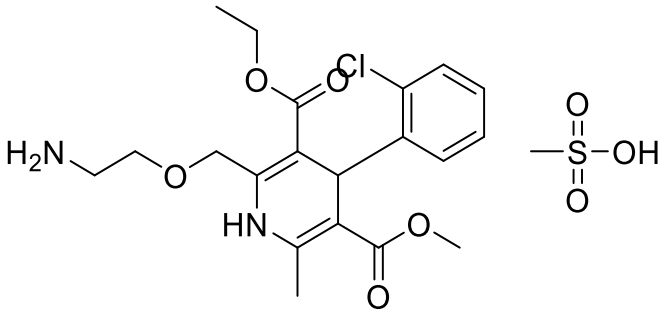

Figure 1.3 Amlodipine besylate structure. (USP 2016)

Amlodipine is a dihydropyridine calcium antagonist (calcium ion channel blocker) that inhibits the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle. Amlodipine binds to both dihydropyridine and nondihydropyridine binding sites. The contractile processes of cardiac muscle and vascular smooth muscle are dependent upon the movement of extracellular calcium ions into these cells through specific ion channels. Amlodipine inhibits calcium ion influx across cell membranes selectively, with a greater effect on vascular smooth muscle cells than on cardiac muscle cells without affecting serum calcium concentration and without significant effect on cardiac conduction. Within the physiologic pH range, amlodipine is an ionized compound (pKa=8.6), and its kinetic interaction with the calcium channel receptor is characterized by a gradual rate of association and dissociation with the receptor binding site, resulting in a gradual onset of effect. Amlodipine is a peripheral arterial vasodilator that acts directly on vascular smooth muscle to cause a reduction in peripheral vascular

resistance and reduction in blood pressure. It's used also for the treatment of chronic stable angina pectoris, and Prinzmetal's variant angina. Amlodipine besylate was approved by the FDA in July 1992 (Faulkner 1986; Kuschnir 1996; Steffen 1999; Agodoa 2001, Nissen 2004; Vincent 2013).

ii. Losartan potassium

The molecular formula for losartan potassium is ($C_{22}H_{23}ClKN_6O$); IUPAC name is 1-((2-(1H-tetrazol-5-yl)-[1,1-biphenyl]-4-yl)methyl)-2-butyl-4-chloro-1H-imidazol-5-yl) methanol, potassium salt, its chemical structure is shown in Figure 1.4.

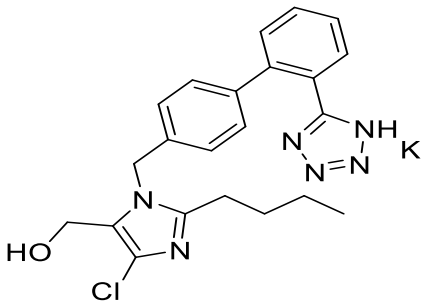

Figure 1.4 Losartan potassium structure. (USP39 2016)

Losartan acts as specific angiotensin II receptors blocker, inhibits the actions of angiotensin II by preventing its formation from angiotensin I, and interferes with the binding of formed angiotensin II to its endogenous receptor. Angiotensin II is the primary vasoactive hormone of the renin-angiotensin system and plays an important role in the pathophysiology of hypertension. Angiotensin II stimulates aldosterone secretion by the adrenal gland. Thus, by blocking the effects of angiotensin II, losartan decreases systemic vascular resistance without a marked change in heart rate. Angiotensin are converting enzyme type (i) receptors are found in many tissues, including vascular smooth muscle and the adrenal gland. Angiotensin converting enzyme type (ii) receptors are also found in many tissues, although their relationship to cardiovascular hemostasis is not known. The affinity

of losartan and its metabolite for the angiotensin converting enzyme type (ii) receptor is about 1000-fold greater than for the angiotensin converting enzyme type (i) receptor. Losartan is the first of a unique class of oral antihypertensive agents; it is selective angiotensin II receptors blocker. Losartan is indicated to treat essential hypertension, diabetic nephropathy, and proteinuria, and has also been used to treat congestive heart failure. Losartan does not result in bradykinin accumulation which causes the cough and angioedema; greater antihypertensive efficacy is achieved by adding a small dose of a diuretic. Although FDA doesn't approve losartan for the treatment of heart failure, it had been shown to lower all-causes of mortality and hospitalization in patients with type (ii) diabetes and chronic heart failure. Losartan was approved by the FDA for the treatment of hypertension in April 1995 and for the treatment of nephropathy in patients with type (ii) diabetes mellitus in September 2002.(Gottlieb et al. 1993; Crozier et al. 1995; Pitt et al. 1997; Dahlof et al. 2002; MERCK 2008; Cozaar 2014).

1.1.3 Amlodipine besylate and Atorvastatine calcium

i. Amlodipine besylate

Reviwed in section 1.1.2.

ii. Atorvastatin calcium

The molecular formula for atorvastatin is $(C_{33}H_{35}FN_2O_5)_2Ca$; IUPAC name is (3R,5R)-7-(2-(4-fluorophenyl)-5-isopropyl-3-phenyl-4-(phenylcarbamoyl)-1H-pyrrol-1-yl)-3,5-dihydroxyheptanoic acid, its chemical structure is shown in Figure 1.5.

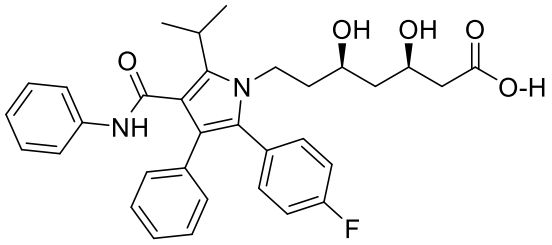

Figure 1.5 Atorvastatin structure. (USP 2016)

Atorvastatin is a selective, competitive hydroxymethylglutaryl-coenzyme A reductase inhibitor. It is primarily used to lower cholesterol and triglycerides in patients with hypercholesterolemia and mixed dyslipidemia, and may also be used for homozygous familial hypercholesterolemia. At the maximum recommended dosage, it has greater low-density lipoprotein -lowering efficacy relative to the maximum recommended dosage of other hydroxymethylglutaryl-coenzyme A reductase inhibitors; this may be explained by its unique structure, long half-life, and hepatic selectivity. In a dose-dependent manner, atorvastatin lowers low-density lipoprotein cholesterol by as much as 60%; oral doses as low as 2.5 mg/day were as effective as lower doses of other hydroxymethylglutaryl-coenzyme A reductase inhibitors. Clinical outcome trials have demonstrated benefits in various populations including the patients treated for the high-risk hypertensives , trials demonstrated reduced cardiovascular events with 80 mg versus 10 mg daily in patients with stable coronary heart disease. Atorvastatin is a selective, competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase which is the rate-limiting hepatic enzyme responsible for converting hydroxymethylglutaryl-coenzyme A to mevalonate, a precursor of sterols including cholesterol. Inhibition of hydroxymethylglutaryl-coenzyme A reductase lowers the amount of mevalonate and subsequently reduces cholesterol levels in hepatic cells. This, in turn, results in upregulation of low-density lipoprotein -receptors and increased hepatic uptake of low-density lipoprotein -cholesterol from the circulation. Atorvastatin ultimately reduces the levels of circulating total cholesterol, low-density lipoprotein -cholesterol, and serum triglycerides. Drug dosage rather than systemic drug concentration correlates better with low-density lipoprotein -cholesterol reduction (Shah et al. 2008; Sever et al. 2003; Bakker et al.1996; Yang et.al 1996; Nawrocki et al. 1995).

1.1.5 Method validation

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for the intended analytical applications. Analytical characteristics used in method validation were outlined in the following section:

1.1.5.1 System suitability

System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being evaluated. In the case of chromatographic procedures, system suitability test is performed from five or six replicate injections of standard working solution. To be sure that the system is stable. The acceptance criteria for system suitability are as follows:

- Relative standard deviation for peak area of the six injections is not more than two (NMT 2).
- Resolution between peaks is not less than two (NLT 2).
- Tailing factors of peaks is not more than two (NMT 2).
- Theoretical plate for per column is not less than two thousand (NLT 2000).

(ICH 1994; Stephan et al. 2002; Gustavo et al. 2007; USP 2016).

1.1.5.2 Linearity and Range

The linearity of an analytical procedure is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Thus, linearity refers to the linearity of the relationship of concentration and response signal (peak area). The goal is to have a model, whether linear or nonlinear, that describes closely the concentration-response relationship. Linearity should be established across the range of the analytical procedure. It should be established initially by visual

examination of a plot of signals as a function of analyte concentration. If there appears to be a linear relationship, test results should be established by appropriate statistical methods (e.g., by calculation of a regression line by the method of least squares). Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity. The correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares should be submitted. The range of an analytical procedure is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated to be determined with a suitable level of precision, accuracy, and linearity using the procedure as written. The range is normally expressed in the same units as test results (e.g., percent, parts per million) obtained by the analytical procedure. The range of the procedure is validated by verifying that the analytical procedure provides acceptable precision, accuracy, and linearity when applied to samples. It is recommended that, for the establishment of linearity, a minimum of five concentrations normally be used. It is also recommended that the following minimum specified ranges should be considered: In case of assay of a drug substance (or a finished product): from 80% to 120% of the test concentration. For content uniformity: a minimum of 70% to 130% of the test concentration, unless a wider or more appropriate range. For dissolution testing: ±20% over the specified range (e.g., if the acceptance criteria for a controlled-release product cover a region from 30%, after 1 hour, and up to 90%, after 24 hours, the validated range would be 10% to 110% of the label claim). (ICH 1994; European Medicines Agency 1995; Piet et al. 1999; Fajgelj et al. 2000; CIPAC 2003; Maxet al. 2007; Gustavo et al. 2007; United Nations Office on Drugs and Crime 2009; FDA 2015; USP 2016).

1.1.5.3. Detection Limit and Quantitation Limit

a) Limit of detection

The detection limit is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. The detection limit is usually expressed as the concentration of analyte (e.g., percentage, parts per billion) in the sample.The detection limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected. In the case of procedures submitted for consideration as official compendial procedures, it is almost never necessary to determine the actual detection limit. In the case of instrumental analytical procedures that exhibit background noise, the Inernational Conference of Hharmonization documents describe a common approach, which is to compare measured signals from samples with known low concentrations of analyte with those of blank samples. The minimum concentration at which the analyte can reliably be detected is established. Typically acceptable signal to- noise ratios are 2:1 or 3:1. Other approaches depend on the determination of the slope of the calibration curve and the standard deviation of responses, which is the method applied in this study.

Limit of detection = 3*(SD/S)

RMSE ≡ SD = the standard deviation of the response signal from regression line

S ≡ slope from linear regression analysis

(ICH 1994; Fajgelj et al. 2000; Stephan et al. 2002; USP 2016).

b) Limit of quantification

The quantitation limit is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. The quantitation limit is expressed as the concentration of analyte (e.g., percentage, parts per billion) in the sample. It is generally determined by the

analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be determined with acceptable accuracy and precision. In the case of procedures submitted for consideration as official compendial procedures, it is almost never necessary to determine the actual quantitation limit. Rather, the quantitation limit is shown to be sufficiently low by the analysis of samples with known concentrations of analyte. In the case of instrumental analytical procedures that exhibit background noise, the Inernational Conference of Harmonization documents describe a common approach, which is to compare measured signals from samples with known low concentrations of analyte with those of blank samples. The minimum concentration at which the analyte can reliably be quantified is established. A typically acceptable signal to noise ratio is 10:1. Other approaches depend on the determination of the slope of the calibration curve and the standard deviation of responses, which is the method applied in this study.

Limit of Quantification = 10 (SD/S)

Root-Mean-Square Error (RMSE) ≡ SD = the standard deviation of the response signal from regression line

S ≡ slope from linear regression analysis

(ICH 1994; Fajgelj et al. 2000; Stephan et al. 2002; Maxet al. 2007; Gustavo et al. 2007; FDA 2015; USP 2016).

1.1.5.4. Specificity and selectivity:

Is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and excipients. [NOTE—Other reputable international authorities such as International Union of Pure and Applied Chemistry and Association of Official Analytical Chemists International, they preferred the term selectivity, for assay it's to provide an exact result, which allows an accurate statement on the content or potency of the

analyte in a sample. In the case of the assay, demonstration of specificity requires that it can be shown that the procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substance or product with appropriate levels of impurities or excipients (placebo) and demonstrating that the assay result is unaffected by the presence of these excipients. when chromatographic procedures are used, representative chromatograms should be presented to demonstrate the degree of selectivity, and peaks should be appropriately labeled.(ICH 1994; Stephan et al. 2002; USP 2016).

1.1.5.5. Accuracy

The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value. The accuracy of an analytical procedure should be established across its range. In the documents of the (ISO), its termed trueness. Accuracy may be determined by application of the analytical procedure to an analyte of known purity (e.g., a Reference Standard) or by comparison of the results of the procedure with those of a second, well-characterized procedure, the accuracy of which has been stated or defined. In the case of the assay of a drug in a formulated product, accuracy may be determined by application of the analytical procedure to synthetic mixtures of the drug product components to which known amounts of analyte have been added within the range of the procedure. If it is not possible to obtain samples of all drug product components, it may be acceptable either to add known quantities of the analyte to the drug product (i.e., “to spike”) or to compare results with those of a second, well characterized procedure, the accuracy of which has been stated or defined. Accuracy is calculated as the percentage of recovery by the assay of the known added amount of analyte in the sample, or as the difference between the mean and the accepted true value, together with confidence intervals. Accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels, covering the

specified range (i.e., three concentrations and three replicates of each concentration). Assessment of accuracy can be accomplished in a variety of ways, including evaluating the recovery of the analyte (percent recovery) across the range of the assay, or evaluating the linearity of the relationship between estimated and actual concentrations. The statistically preferred criterion is that the confidence interval for the slope be contained in an interval around 1.0, (not less than 0.997). (ICH 1994; European Medicines Agency 1995; Piet et al. 1999; Fajgelj et al. 2000; Stephan et al. 2002; CIPAC 2003; Maxet al. 2007; Gustavo et al. 2007; FDA 2015; USP 2016).

1.1.5.6. Precision (Repeatability and/or Reproducibility)

The precision of an analytical procedure is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. The precision of an analytical procedure is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements. Precision may be a measure of either the degree of reproducibility or of repeatability of the analytical procedure under normal operating conditions. In this context, reproducibility refers to the use of the analytical procedure in different laboratories, as in a collaborative study. Intermediate precision (also known as ruggedness) expresses within-laboratory variation, as on different days, or with different analysts or equipment within the same laboratory. Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time using the same analyst with the same equipment. The precision of an analytical procedure is determined by assaying a sufficient number of aliquots of a homogeneous sample to be able to calculate statistically valid estimates of standard deviation or relative standard deviation (coefficient of variation). Assays in this context are independent analyses of samples that have been carried through the complete analytical procedure from

sample preparation to final test result. It is recommend that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e., three concentrations and three replicates of each concentration) or using a minimum of six determinations at 100% of the test concentration.
(ICH 1994; ; Stephan et al. 2002; Gustavo et al. 2007; USP 2016).

1.1.5.7. Robustness

Robustness is a measure of the performance of a method when small, deliberate changes are made to the method conditions, these should be suitably controlled, or a precautionary statement should be included in the procedure to ensure that the validity of the analytical procedure is maintained. Typical variations are the pH of the mobile phase, the mobile phase composition, different lots or suppliers of columns, the temperature, and the flow rate. (ICH 1994; ; Stephan et al. 2002; Gustavo et al. 2007; USP 2016).

1.2 Literature review

1.2.1 Hydrochlorothiazide and Valsartan

Ankit et al. (2010) developed a spectrophotometric method for determination of valsartan and hydrochlorothiazide simultaneously in tablet dosage forms, using methanol as solvent. The detection wavelengths were 231.5 and 270.5 nm, respectively; linearities of their procedure were within the concentration range of 2–20 μg/mL for both valsartan and hydrochlorothiazide; limits of detection were 0.628 μg/ml and 0.413 μg/ml, respectively; while limits of quantitation were1.902 μg/ml and 1.251 μg/ml, respectively.

Sunil et al.(2011) developed a spectrophotometric method for determination of valsartan and hydrochlorothiazide simultaneously in tablet dosage forms using 0.1M NaOH as solvent. The detection wavelengths were 248.5nm and 271nm, respectively; linearities of their procedure were within the concentration range of 0.5-3.5 mg/ml and 0.2-1.4 mg/ml for valsartan and hydrochlorothiazide, respectively; limits of detection were 0.69 mg/ml and 0.13mg/ml, respectively; while limits of quantitation were 1.83μg/ml and 0.42 mg/ml, respectively.

Karunandhi and Sivasubramanian (2011) developed a spectrophotometric method for determination of valsartan and hydrochlorothiazide simultaneously in tablet dosage forms, using 0.1M NaOH as solvent. The detection wavelengths were 216 and 228 nm, respectively; linearities of their procedure were within the concentration range of 0.5–3 μg /mL for both valsartan and hydrochlorothiazide; limits of detection were 0.51 mg/ml and 0.62 μg/ml, respectively.

Namrata et al. (2012) developed a first derivative spectrophotometric method for determination of valsartan and hydrochlorothiazide simultaneously in tablet dosage formsusing 0.1M NaOH as solvent. The detection wavelengths were

270.60 nm and 250.2 nm, respectively; linearities of their procedure was within the concentration range of 4-20 μg/ml and 2-14 μg/ml, for valsartan and hydrochlorothiazide respectively. The limits of detection were 1.157 μg/ml and 0.634 μg/ml, respectively; while the limits of quantitation were 3.50μg/ml and 1.92 μg/ml, respectively.

Nevin (2002) developed a first derivative spectrophotometric method for determination of valsartan and hydrochlorothiazide simultaneously in tablet dosage forms using 0.1M NaOH as solvent. The detection wavelengths were 227.8 and 276.5nm, respectively; linearities of their procedure were within the concentration range of 2.0‑18.0 μg/ml and 1.5‑15.0 μg/ml for valsartan and hydrochlorothiazide, respectively; limits of detection and limits of quantitation were not recorded in this work.

Kadam and Bari (2007) developed a high-performance thin-layer chromatographic (HPTLC) method for simultaneous analysis of valsartan and hydrochlorothiazide in tablet formulations, using precoated silica gel G 60 F254 HPTLC plates mobile phase composed of chloroform–ethyl acetate–acetic acid, (5:5:0.2). The analytes were densitometrically detected at 248 nm. The retention factors of valsartan and hydrochlorothiazide were 0.27 and 0.56, respecti-vely. The linear range was 800–5600 ng per spot for valsartan and 125–875 ng per spot for hydrochlorothiazide. Limit of detection and limit of quantitation were not recorded in this work.

Maher (2012) developed HPLC-UV simultaneouse method for determination of valsartan and hydrochlorothiazide, using column C18 (250x4.6 mm, 5μm); injection volume was 20 μL; the mobile phase was ammonium acetate buffer pH 5.6 and acetonitrile, using gradient elution with flow rate of 1.5 ml/min and eluents were monitored at 265 nm. Linearity ranges were 2.5–32μg/ml and 17.5-224μg/ml

for valsartan and hydrochlorothiazide, respectively. Limits of detection were 0.008μg/ml and 0.0375 μg/ml, respectively; while limits of quantitation were 0.075 μg/ml and 0.064 μg/ml, respectively.

Mamdouh et al.(2012) developed simultaneouse HPLC-UV method for determination of valsartan and hydrochlorothiazide, using column C18 (150x4.6 mm, 5μm), injection volume was 50 μL, the mobile phase was phosphate buffer pH 2.9 acetonitrile and methanol (50:40:10) using isocratic elution with flow rate 1.4 ml/min and eluents were monitored at 225 nm. Linearity ranges were 12-36 μg/mL and 2-9μg/mL for valsartan and hydrochlorothiazide, respectively; limits of detection and limits of quantitation were notrecorded.

Ashok (2016) developed simultaneouse HPLC-UV method for determination of valsartan and hydrochlorothiazide, using column C18 (150x4.6 mm, 5μm) maintained at 25ºC, injection volume was 20 μL, the mobile phase was 0.25 ml/L triethylamine (pH 3.0), methanol and acetonitrile (50:38:37), using isocratic elution with flow rate of 1.5 ml/min and eluents were monitored at 265 nm. Linearity ranges were 1.25-64.00 μg/ml and 0.195-10.00 μg/ml, limits of detection were 0.253 and 0.0226 μg/ml, respectively; while limits of quantitation were 0.767 and 0.068 μg/ml for valsartan and hydrochlorothiazide, respectively.

Antil et al.(2013) developed UPLC-UV simultaneous method for determination of valsartan and hydrochlorothiazide, using column C18 (50x2.1 mm, 3.5μm) maintained at 25ºC, injection volume was 20 μL, the mobile phase was 0.1% triethylamine : methanol (75:25), using isocratic elution with flow rate 0.6 ml/min and eluents were monitored at 225 nm. Linearity ranges were 56-104 μg/ml and 7-13 μg/ml for valsartan and hydrochlorothiazide, limits of detection were 0.8 and

0.12 μg/ml, while limits of quantitation were 2.4 and 0.36 μg/ml for valsartan and hydrochlorothiazide respectively.

1.2.2 Amlodipine besylate and losartan potassium

Sunil et al.(2012) developed simultaneous spectrophotometric method for determination of losartan potassium and amlodipine besylate, using methanol as solvent, the detection wavelengths were 247 nm and 354nm, respectively; linearity of the procedure was within the concentration range of 2-20 μg/mL for both components. Limits of detection and limits of quantitation were not recorded.

Priyanka et al.(2009) developed simultaneous spectrophotometric method for determination of losartan potassium and amlodipine besylate using methanol as solvent, the detection wavelengths were 208 nm and 237.5 nm, respectively; linearity of procedure was within the concentration range 2-20 μg/ml for both components. The imits of detection and limits of quantitation were not recorded.

Ramya et al.(2012) developed simultaneous HPLC-UV method for determination of valsartan and hydrochlorothiazide using column C18 (250x4.6mm,5μm) maintained at 25ºC, injection volume was 20 μL, the mobile phase was phosphate buffer (pH 3) : acetonitrile (1:1) using isocratic elution with flow rate 1ml/min and eluents were monitored at 230 nm, linearity ranges were 0.125 -0.75 μg/ml and 1.25-7.5 μg/ml, limits of detection were 0.0009 and 0.0027 μg/ml for amlodipine and losartan while limits of quantitation were 0.03 and 0.1 μg/ml respectively.

Kumari et al.(2013) developed simultaneous HPLC-UV method for determination of valsartan and hydrochlorothiazide using column C18 (250x4.6mm,5μm) maintained at 25ºC, injection volume was 20 μL, the mobile phase was triethylamin (pH3) : acetonitrile (70:30) using isocratic elution with flow rate of 1ml/min eluents were monitored at 246 nm, linearity ranges were 0.01 -0.03μg/ml

and 0.1 -0.3 μg/ml, limits of detection were 0.000069 and 0.00063 μg/ml, while limits of quantitation were 0.00023 and 0.0021 μg/ml for amlodipine and losartan, respectively.

Krishna et al.(2013) developed HPLC-UV simultaneous method for determination of valsartan and hydrochlorothiazide using column C18 (150x4.6mm,5μm) maintained at ambient temperature, injection volume was 20 μL, the mobile phase was phosphate buffer (pH3.7) : acetonitrile (70:30) using isocratic elution with flow rate of 1ml/min and eluents were monitored at 237 nm. Linearity ranges were 1.25-7.5 μg/ml and 12.5-75 μg/ml, limits of detection were 0.041and 0.080 μg/ml, while limits of quantitation were 0.135 and 0.264 μg/ml for amlodipine and losartan respectively.

Priyanka et al. (2009) developed HPLC-UV simultaneous method for determination of valsartan and hydrochlorothiazide using column C18 (250x4.6mm,5μm) maintained at ambient temperature, injection volume was 20 μL, the mobile phase was 0.02% triethylamin (pH 2.5): acetonitrile (60:40) using isocratic elution with flow rate of 1ml/min and eluents were monitored at 226 nm. Linearity ranges were 5-50 μg/ml and 50-500 μg/ml for amlodipine and losartan respectively; limits of detection and limits of quantitation were not reported.

Carlos et al.(2009) developed HPLC-UV simultaneous method for determination of valsartan and hydrochlorothiazide using column C18 (150x4.6mm,5μm), injection volume was 20 μL, the mobile phase was phosphate buffer (pH3) and acetonitrile using gradient elution with flow rate of 1ml/min and eluents were monitored at 237 nm. Linearity ranges were 1.4 - 4.2 μg/ml and 20 - 60 μg/ml for amlodipine and losartan, respectively.

Murali et al.(2014) developed simultaneous HPLC-UV method for determination of valsartan and hydrochlorothiazide using column C18 (150x4.6mm,5μm),

injection volume was 20 µL, the mobile phase was phosphate buffer (pH4) and acetonitrile (40:60), using isocratic elution with flow rate of 1ml/min and eluents were monitored at 225 nm. Linearity ranges were 30- 90 µg/ml and 300-900 µg/ml, limits of detection 2.903 and 2.941 µg/ml and limit of quantitation 9.675 and 9.8 µg/ml for amlodipine and losartan, respectively.

1.2.3 Amlodipine besylate and Atorvastatine calcium

Hany et al.(2013) developed a simultaneous spectrophotometric method for determination of amlodipine besylate and atorvastatin calcium in tablet dosage forms, detection wavelengths were 238nm and 266 nm and linearity ranges were 4–40 µg/mL and 8–32 µg/ml, respectively. Limit of detection and limit of quantitation were not recorded in this work.

Kapil et al. (2013) developed a simultaneous spectrophotometric method for determination of amlodipine besylate and atorvastatin calcium using phosphate buffer (pH 6.8) as solvent, the detection wavelengths were 369nm and 240 nm respectively, linearity ranges were 10–50 µg/mL and 5–25 µg/ml, respectively. Limits of detection and limits of quantitation were not recorded in this work.

Juyal et al.(2008) developed a simultaneous spectrophotometric method for determination of amlodipine besylate and atorvastatin calcium using methanol: water (1:1) as solvent, the detection wavelengths were 363nm and 245nm respectively, and linearity ranges were 14–26 and 7–13 µg/ml, respectively. Limits of detection and limits of quantitation were not recorded in this work.

Devi, and Ramakrishna (2010) developed a simultaneous spectrophotometric method for determination of amlodipine besylate and atorvastatin calcium using methanol as solvent, the detection wavelengths were 238.8nm and 246nm respectively, and linearity ranges were 0.5–30 µg/mL for both components. Limits

of detection were 0.028 µg/mL and 0.054 µg/ml, while limits of quantitation were 0.0.086 µg/mL and 0.163 µg/ml, respectively.

Smita et al. (2011) developed a first drivative simultaneous spectrophotometric method for determination of amlodipine besylate and atorvastatin calcium, using methanol : water (1:1) as solvent, the detection wavelengths were 250nm and 241nm respectively, linearity ranges of their concentration were 0–7 µg/mL and 0–14 µg/ml, limits of detection were 0.21 µg/mL and 0.29 µg/ml, while limits of quantitation were 0.6 µg/mL and 0.75 µg/ml, respectively.

Babikir and Elsaman (2016) developed simultaneous HPLC-UV method for determination of amlodipine besylate and atorvastatin calcium using column C18 (250x4.6 mm, 5µm) mentained at 30ºC, injection volume was 20 µL, the mobile phase was acetate buffer (pH 4) : Acetonitrile (1:1) , isocratic elution with flow rate of 1.5 ml/min and eluents were monitored at 240 nm. Linearity ranges were 2.5 µg/ml -40 µg/ml and 10.0 µg/ml -160 µg/ml respectively, limits of detection were 0.0047 µg/ml and 0.0035 µg/ml respectively while limits of quantitation were 0.014 µg/ml and 0.0102 µg/ml for Amlodipine besylate and Atorvastatin calcium, respectively.

Hafez et al. (2014) developed simultaneous HPLC-UV method for determination of amlodipine besylate and atorvastatin calcium using column C18 (250x4.6 mm, 2.6µm) maintained at 40ºC, injection volume was 20 µL, the mobile phase was phosphate buffer (pH 5.5) : Acetonitrile (65:35) , isocratic elution with flow rate of 1.2 ml/min and eluents were monitored at 240 nm. Linearity ranges were 5.18 µg/ml -15.54 µg/ml and 5.26 µg/ml -15.78 µg/ml respectively, limits of detection were 0.16 µg/ml and 0.17 µg/ml respectively while limits of quantitation were 0.48

μg/ml and 0.52 μg/ml for Amlodipine besylate and Atorvastatin calcium, respectively.

Majdi and Agha (2015) developed simultaneous HPLC-UV method for determination of amlodipine besylate and atorvastatin calcium using column C18 (250x4.6 mm, 5μm) at room temerature, injection volume was 20 μL, the mobile phase was phosphate buffer (pH 5.5) : Acetonitrile, gradient elution with flow rate of 1.0 ml/min and eluents were monitored at 240 nm. Linearity ranges were 5-30 μg/ml for Amlodipine besylate and Atorvastatin calcium, limits of detection were 0.08 and 0.05 μg/ml, while limits of quantitation were 0.27 and 0.17 μg/ml, respectively.

Manzoor et al. (2012) developed simultaneous HPLC-UV method for determination of amlodipine besylate and atorvastatin calcium, using column C18 (250x4.6 mm, 5μm) at 30^{o}C, injection volume was 20 μL, the mobile phase was ammonium acetate buffer (pH 6.1) : Acetonitrile (45:55) , gradient elution with flow rate 1.2 ml/min, an eluents were monitored at 240 nm. linearity range was 5-15μg/ml for amlodipine besylate and atorvastatin calcium, limits of detection were 0.025 and 0.029 μg/ml, respectively; while limits of quantitation were 0.088 μg/ml and 0.076 μg/ml, respectively.

Imre et al. (2013) developed simultaneous HPLC-UV method for determination of amlodipine besylate and atorvastatin calcium using column C18 (150x4.6 mm, 3μm) at 30^{o}C, injection volume was 10 μL, the mobile phase was potassium dihydrogen phosphate 0.01M, and Acetonitrile using gradient elution with flow rate of 1.0 ml/min, amlodipine besylate and atorvastatin calcium were monitored at 240 nm. Linearity range was 3-15 μg/ml for amlodipine besylate and

atorvastatin calcium; limits of detection and limits of quantitation were not specified in this work.

Sasmita et al. (2010) developed simultaneous HPLC-UV method for determination of amlodipine besylate and atorvastatin calcium using column C18 (250x4.6 mm, 5μm) at ambient temperature, injection volume was 20 μL, the mobile phase was ammonium acetate buffer (pH 4) : Acetonitrile (40:60) , isocratic elution with flow rate 1.0 ml/min, and eluents were monitored at 240 nm. Linearity range was 30-70 μg/ml for amlodipine besylate and 60-140 μg/ml atorvastatin calcium, and limits of detection were 0.35 and 0.40 μg/ml, respectively; while limits of quantitation were 1.05 and 1.2 μg/ml respectively.

Mohamed, et al. (2013) developed simultaneous HPLC-MS method for determination of amlodipine besylate and atorvastatin calcium using Synergi polar column (150 mm×4.6 mm, 4 μm) at 30°C, injection volume was 20 μL, the mobile phase was water/methanol (14:86%, v/v) adjusted by trichloroacetic acid to pH 3.2, elution was isocratic with flow rate of 0.5 ml/min, and eluents were monitored with mass detector operated in a positive ion mode. Linearity range was 0.2-20 μg/ml for amlodipine besylate and 1.5-150 ng/ml for atorvastatin calcium, and limits of quantitation were 0.2 and 1.5 μg/ml, respectively.

Hossein et al. (2015) developed simultaneous HPLC-MS method for determination of amlodipine besylate and atorvastatin calcium column C18 (150 mm×4.6 mm, 4 μm) at 30°C, injection volume was 20 μL, the mobile phase was ammonium acetate buffer pH3: acetonitrile (30:7), elution was isocratic with flow rate 0.15 ml/min, and eluents were monitored with mass detector operated in a positive ion mode. Linearity ranges was 0.1-10 μg/ml for amlodipine besylate and 0.2-20 μg/ml atorvastatin calcium, and limits of quantitation were 0.1 and 0.2 μg/ml respectively.

1.2.4 Hydrochlorothiazide, Amlodipine besylate and losartan potassium

D. Nagavalli et al. (2010) developed a simultaneous spectrophotometric method for determination of losartan potassium, amlodipine besilate and hydrochlorothiazide using methanol as solvent, the maximum absorbance was measured at wavelength range 230.5 - 350.2nm, linearity of procedure was within the concentration ranges of 8–4015μg/ml, 1–515μg/ml and 3–15μg/ml, respectively. Limits of detection and limits of quantitation were not reported.

Wankhede et al. (2010) developed a simultaneous spectrophotometric method for determination of amlodipine besilate, losartan potassium and hydrochlorothiazide using methanol as solvent; the detection wavelengths were 236.5, 254 and 271 nm, respectively, and linearities of procedure were within the concentration ranges 5-25 μg/ml, 10-50 μg/ml and 5-25 μg/ml, respectively. They developed also an HPLC method for determination of same drug using column C18 (250x4.6 mm, 5μm) at ambient temperature. The injection volume was 20 μL, the mobile phase was phosphate buffer (pH 3.7) : Acetonitrile (57:43) , with isocratic elution flow rate 1.0 ml/min, and eluents were monitored at 232 nm. Linearity ranges were 2-14 μg/ml, 20-140μg/ml and 5-40μg/ml for amlodipine besylate, losartan potassium and hydrochlorothiazide, respectively. Limits of detection and limits of quantitation were not reported for both methods.

Babikir, et al. (2015) developeed a simultaneous HPLC-UV method for determination of amlodipine besilate, losartan potassium and hydrochlorothiazide using C18 column (250x4.6 mm, 5μm) at ambient temperature, injection volume was 20 μL, the mobile phase was phosphate buffer (pH 3.0) ,Acetonitrile and methanol, mixed in ratio of 5:3:3.5, respectively; with flow rate 1.0 ml/min; eluents were monitored at 240 nm. Linearity ranges of hydrochlorothiazide and amlodipine were 10μg/ml-120μg/ml and 2μg/ml-48μg/ml, respectively.

Anandkumar et al. (2013) developeed a simultaneous HPLC-UV method for determination of amlodipine besilate, losartan potassium and hydrochlorothiazide using CN column (250x4.6 mm, 5µm) at ambient temperature, injection volume was 20 µL, the mobile phase was phosphate buffer (pH 2.7) ,Acetonitrile and water; gradient elution was used with flow rate 1.0 ml/min; eluents were monitored at 230 nm. Linearity ranges of hydrochlorothiazide, amlodipine and losartan were 12.5µg/ml-62.5µg/ml, 2.5µg/ml-12.5µg/ml and 50 µg/ml -250µg/ml, limits of detection were 0.03µg/ml, 0.03µg/ml and 0.18µg/ml, while limits of quantitation were 0.1µg/ml, 0.1µg/ml and 0.228µg/ml, respectively.

Savita et al. (2014) developed a simultaneous HPLC-UV method for determination of amlodipine besilate, losartan potassium and hydrochlorothiazide using C18 column (250x4.6 mm, 5µm) at ambient temperature, injection volume was 20 µL, the mobile phase was methanol: water in the ratio of 95:5, isocratic elution was used, with flow rate of 0.8 ml/min and eluents were monitored at 230 nm. Linearity ranges of hydrochlorothiazide, amlodipine and losartan were 2.5µg/ml -15µg/ml, 1µg/ml -6µg/ml and 10µg/ml -60µg/ml, limits of detection were 0.8µg/ml, 0.4µg/ml and 3µg/ml, while limits of quantitation were 1.2 µg/ml, 0.8 µg/ml and 8µg/ml, respectively.

Jayaseelan et al. (2010) developed a simultaneous HPLC-UV method for determination of amlodipine besilate, losartan potassium and hydrochlorothiazide using C18 column (250x4.6 mm, 5µm) at ambient temperature, injection volume was 20 µL, the mobile phase was phosphate buffer (pH 7.0), methanol and acetonitrile in ratio of 60:20:20, isocratic elution with flow rate 1.0 ml/min and eluents were monitored at 238 nm. The linearity ranges of hydrochlorothiazide, amlodipine and losartan were 27.84µg/ml-41.76µg/ml, 50µg/ml-75µg/ml, and 200µg/ml-300µg/ml, limits of detection were 0.139, 0.051µg/ml and 1.522µg/ml,

while limits of quantitation were 0.421µg/ml, 0.156µg/ml and 4.612µg/ml, respectively.

Surekha et al. (2014) developed a simultaneous HPLC-UV method for determination of amlodipine besilate, losartan potassium and hydrochlorothiazide using C18 column (100x4.6 mm, 5µm) at 40^{o}C, injection volume was 20 µL, the mobile phase was 0.1% phosphoric acid and a mixture of methanol :acetonitrile (5:95), gradient elution with flow rate of 1.5 ml/min was used, and eluents were monitored at 217 nm. The linearity ranges of hydrochlorothiazide, amlodipine and losartan were 0.02µg/ml-0.03µg/ml, 0.008µg/ml-0.012µg/ml and 0.08µg/ml-0.12µg/ml, respectively. Limits of detection and limits of quantitation were not reported.

Gurlin et al. (2014) developed a simultaneous HPLC-UV method for determination of amlodipine besilate, losartan potassium and hydrochlorothiazide using C8 column (150x4.6 mm, 5µm) at ambient temperature, injection volume was 20 µL, the mobile phase was phosphate buffer (pH 7.0), and acetonitrile (7:5), isocratic elution with flow rate 1.0 ml/min was applied, and eluents were monitored at 254nm. The linearity ranges of hydrochlorothiazide, amlodipine and losartan were 62µg/ml -188µg/ml, 25µg/ml -75µg/ml, and 25µg/ml -75µg/ml, limits of detection were 0.139µg/ml, 0.051µg/ml and 1.522µg/ml, while limits of quantitation were 0.421µg/ml, 0.156µg/ml and 4.612µg/ml, respectively.

Anandkumar et al. (2015) developed a simultaneous HPLC-MS method for determination of amlodipine besilate, losartan potassium and hydrochlorothiazide using C18 column (50x2.1 mm, 1.7µm) at ambient temperature, injection volume was 2µL, the mobile phase was 1% ammonium acetate (pH 2.6) and acetonitrile, gradient elution with flow rate 0.4 ml/min was applied and eluents were monitored

at 254 nm. The linearity ranges of hydrochlorothiazide, amlodipine and losartan were 125ng/ml - 750ng/ml, 50ng/ml - 300ng/ml, and 500ng/ml - 3000ng/ml, limits of detection were 0.6ng/ml, 0.1ng/ml and 2ng/ml, while limits of quantitation were 1ng/ml, 1ng/ml and 5ng/ml, respectively.

1.2.5 Hydrochlorothiazide, Amlodipine besylate and Valsartan

Varsha et al. (2012) developed asimultaneous spectrophotometric method for determination of amlodipine besylate, hydrochlorothiazide and valsartan using methanol as solvent, the selected wavelengths were 359 nm, 317 nm and 250 nm, the linear concentration ranges were 5µg/ml -25µg/ml, 10µg/ml -50µg/ml and 5µg/ml -25µg/ml, limits of detection were 0.51µg/ml, 0.91µg/ml and 1.57µg/ml, while limits of quantitation were 1.68µg/ml, 3.02µg/ml and 4.77µg/ml respectively.

Jothieswari et al. (2010) developed a simultaneous spectrophotometric method for determination of amlodipine besylate, valsartan and hydrochlorothiazide using methanol as solvent, the selected wavelengths were 239 nm, 250 nm and 272 nm, the linear concentration ranges were 1µg/ml -32µg/ml, 4µg/ml -40µg/ml and 2µg/ml -20µg/ml, limits of detection were 0.1µg/ml, 0.3µg/ml and 0.2µg/ml, while limits of quantitation were 0.3µg/ml, 0.9µg/ml and 0.6µg/ml, respectively.

Ananda et al. (2011), developed asimultaneous spectrophotometric method for determination of amlodipine besylate, valsartan and hydrochlorothiazide using methanol:water (1:1) as solvent, the selected wavelengths were 365 nm, 250 nm and 315 nm, the linear concentration ranges were 1µg/ml–32µg/ml, 4µg/ml-40µg/ml and 2µg/ml–20µg/ml, limits of detection were 0.2µg/ml, 0.3µg/ml and 0.25µg/ml, while limits of quantitation were 0.55µg/ml, 0.9µg/ml and 0.75µg/ml, respectively.

Nikam et al. (2010) developed a simultaneous first derivative spectrophotometric method for determination of valsartan, amlodipine besylate and hydrochlorothiazide using methanol:water (7:3) as solvent, the selected wavelengths were 245 nm, 265 nm and 279 nm for valsartan, amlodipine besylate and hydrochlorothiazide, the linear concentration ranges were 8µg/ml-80µg/ml, 1µg/ml–10µg/ml and 2µg/ml – 20µg/ml, limits of detection were 0.46µg/ml, 0.2µg/ml and 0.13µg/ml, while limits of quantitation were 1.3µg/ml, 0.63µg/ml and 0.42µg/ml respectively.

Silvana et al. (2011) developed a simultaneous HPLC-UV method for determination of amlodipine, hydrochlorothiazide and valsartan using C18 column (250x4.6 mm, 5µm) at 30ºC, injection volume was 20 µL, the mobile phase was phosphate buffer (pH 5.5): methanol (38:62), isocratic elution with flow rate 1.0 ml/min was applied, and eluents were monitored at 234 nm. The linearity ranges of amlodipine, hydrochlorothiazide and valsartan were 7µg/ml –13µg/ml, 17.6µg/ml – 32.8µg/ml, and 226.2µg/ml–420.2µg/ml, respectively; limits of detection and limits of quantitation were not reported.

Samya et al. (2012) developed a simultaneous HPLC-UV method for determination of amlodipine, hydrochlorothiazide and valsartan using C18 column (150x4.6 mm, 5µm) at 30ºC, injection volume was 20 µL, the mobile phase was phosphate buffer (pH 2.8): acetonitril (60:40), isocratic elution with flow rate 0.8 ml/min was applied and eluents were monitored at 227 nm. The linearity ranges of amlodipine, hydrochlorothiazide and valsartan were 4µg/ml–28µg/ml, 1µg/ml–12µg/ml, and 5µg/ml–40µg/ml, limits of detection were 1.04µg/ml, 0.39µg/ml and 1.4µg/ml, while limits of quantitation were 3.16µg/ml, 0.81µg/ml and 4.3µg/ml, respectively.

Shankar et al. (2014) developed a simultaneous LC-MS internal standard method for determination of amlodipine, hydrochlorothiazide and valsartan using C18 column (50x2.1 mm, 5µm) at 30^{o}C, injection volume was 20 µL, the mobile phase was 0.1% formic acid: acetonitril (1:1), isocratic elution with flow rate 0.8 ml/min was applied, and eluents were monitored at 227 nm. The linearity range was 1 - 1000ng/ml for amlodipine, hydrochlorothiazide and valsartan.

Rasha et al. (2013) developed a simultaneous HPLC-UV method for determination of amlodipine, hydrochlorothiazide and valsartan using C8 column (250x4.6 mm, 5µm) at ambient temperature, injection volume was 20 µL, the mobile phase was 0.025M phosphoric acid: acetonitril using gradient elution with 1 ml/min flow rate, eluents were monitored at 238nm for amlodipine and 225 nm for both hydrochlorothiazide and valsartan. The linearity range of amlodipine, hydrochlorothiazide and valsartan were 5µg/ml –200µg/ml, 10µg/ml –200µg/ml, and 5µg/ml –200µg/ml, limits of detection were 0.26µg/ml, 0.12µg/ml and 0.24µg/ml, while limits of quantitation were 0.85µg/ml, 0.4µg/ml and 0.8µg/ml respectively.

Ritesh et al. (2012) developed asimultaneous HPLC-UV method for determination of amlodipine, hydrochlorothiazide and valsartan using C8 column (250x4.6 mm, 5µm) at ambient temperature, injection volume was 20 µL, the mobile phase was ammonium formate (pH3.5) and acetonitril using gradient elution with 1 ml/min flow rate, eluents were monitored at 238nm for amlodipine and 225 nm for both hydrochlorothiazide and valsartan. The linearity ranges of amlodipine, hydrochlorothiazide and valsartan were 6ng/ml –200ng/ml, 5ng/ml – 400ng/ml, and 50ng/ml –4000ng/ml, limits of detection were 2ng/ml, 2ng/ml and 7ng/ml, while limits of quantitation were 6ng/ml, 5ng/ml and 20ng/ml, respectively.

1.3 Objectives and Research Purposes:

In the pharmaceutical industry when a new drug has been developed, the main concern after its formulation is to develop and validate an analytical method which can determine the active ingredients quantitatively, some times they may use an advanced instruments, like LC-MS, which is not available in all quality control laboratories of the pharmaceutical factories in The Sudan, or they use two different methods for the assay of different active in the same combination as in USP method for the assay of flow tab ingredients.

The main objective of this research work is to develop and validate assay methods using, instead, simple common instruments, like HPLC-UV chromatographs, which are available in most of these laboratories. The developed methods should be simple, precise, accurate and selective even when the drug contains more than one active ingredient. The selected multi active ingredient drugs are those widely used by millions of hypertensive patients. The intension is also to use, in these liquid chromatographic methods, the simple isocratic elution instead of the more complex gradient elution which may require experienced persons, and experience deficiency may produce wrong results. The selected combinations of antihypertensive drugs may have official method to analyse each active ingredient separately, til now there is no official method for the simultaneous determination for the selected combinations:

i) Amlodipine besylate and losartan potassium.

ii) Hydrochlorothiazide and Valsartan.

iii) Amlodipine besylate and Atorvastatine calcium.

iv) Hydrochlorothiazide, Amlodipine besylate and losartan potassium.

v) Hydrochlorothiazide, Amlodipine besylate and Valsartan.

2.1 Chemicals

- Atorvastatine Calcium (Indo co Remedies Limited - India)
- Amlodipine Besylate (Ranbaxy Laboratories Limited - India)
- Losartan Potassium (Ranbaxy Laboratories Limited - India)
-Valsartan (Ranbaxy Laboratories Limited - India)
- Hydrochlorothiazide (Ranbaxy Laboratories Limited - India)
- formulated tablets (Local market - Riyadh - KSA)
- All excepients were obtained from Blue Nile Pharmaceutical Factory - Sudan - Khartoum).
- Acetonitrile -HPLC grade – Scharlau-Spain.
- Methanol -HPLC grade – Scharlau-Spain.
- Formic acid -HPLC grade – Scharlau-Spain.
- Water -HPLC grade

2.2 Instruments

* High Performance Liquid Chromatography(HPLC)

Company: Shimadzu Corporation

Origin: Tokyo - Japan

Model: LC- 2010A HT

Serial No.:C21245107160LP

* High Performance Liquid Chromatography(HPLC)

Company: Shimadzu Corporation

Origin: Tokyo - Japan

Model: Prominamce

-DAD- Sr.No. L20154807000AE

- Online degasser - Sr.No. L20254813612CR

- Quaternary pump - Sr.No. L2010482018 AE

* Phenyl hexyle Colum, (150mmx4.6mm I.D, 5 µm)

Company: Thermo Scientific

Origin:USA

Serial No.:0503428B

* Neucleodur polaratic50/2 Colum, (50mmx2mm I.D, 1.8 µm)

Company: Macherey Nagel Co.LTD

Origin:USA

Serial No.:E 12010958

* Analytical balance

Company: Dietikon

Origin: Switzerland

Model:360 ES

Serial No.: 4600313

* Ultrasonic bath

Company: Jeiotech

Origin:Japan

Model:UC-10

Serial No.:02(2627-3811)

2.3 Glassware and apparatus

- 50-ml volumetric flask – Clas -A - Germany.
- 100-ml volumetric flask – Clas -A - Germany.
- 250-ml volumetric flask – Clas -A - Germany.
- 10-ml graduated pipette – Clas -A - Germany.
- Glass funnel – 6 cm diameter – Clas -A - Germany.
- Mortar - porcelane - 80 cm^3 volume - Germany.
- Buchner system – quick fit – 1.25L volume - Germany.
- Syringe filter - nylon, 0.22micrometer porous- Germany.
- Nylon membrane filter 0.45micrometer porous - Germany

2.4 Procedures

2.4.1 Hydrochlorothiazide & Valsartan

2.4.1.1 Optimized chromatographic conditions

Phenyl hexyl column (150mm × 4.6 mm, 3 µm), and simple isocratic elusion, were used(one pump required) with flow-rate of 0.8 ml/min, both active ingredients were detected at 275 nm, injection volume was 20µl (universal loop) and analysis temperature was 25^{o}C (ambient temperature).

2.4.1.2 Buffer (1% v/v formic acid)

1000- ml volumetric flask was half filled with deionised water, 10 ml of formic acid was added to the flask, and the volume was completed to the mark with deionised water.

2.4.1.3 Mobile phase

Mixture of methanol and buffer was prepared in 75:25 ratio, respectively. the mixture was shaken, filtered with vacuum filtration pump through 0.45µm nylon membrane filter, and then transferred to solvent reservoir and sonicated for 5 min.

2.4.1.4 Standard Stock Solution

Valsartan (0. 16 g) and hydrochlorothiazide (0. 0125 g) were weighed accurately and transferred quantitatively to the same 100-ml volumetric flask. The flask was half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature, the volume was completed to the mark with the same solvent.

2.4.1.5 System Suitability

Subsequent dilutions were made from the stock solution with mobile phase to give the concentrations of 6.25µg/ml solutions for hydrochlorothiazide and 40µg/ml solutions of valsartan. System suitability solution was injected six times.

2.4.1.6 Linearity, LOD and LOQ

Subsequent dilutions were made from the stock solution with mobile phase to give concentrations of 2.5, 3.75, 5, 6.25,7.5 ,8.75 and 10 µg/ml hydrochlorothiazide solutions and 5,7.5,10,12.5,15,17.5 and 20 µg/ml valsartan solutions. Each solution was injected

three times and results were collected, LOD and LOQ were calculated from the linear regression analysis.

2.4.1.7 Specificity

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 6.25µg/ml for hydrochlorothiazide and 40µg/ml for valsartan. This solution was injected six times.

(b) Placebo

A placebo equivalent to average weight of one tablet was transferred to 100-ml volumetric flask. The flask was half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature, the volume was completed to the mark with the same solvent. Subsequent dilutions were made with mobile phase similar to those made for standard preparation.

(c) Sample

A placebo equivalent to that of one tablet's weight was transferred to 100 ml volumetric flask; 0. 16 g of Valsartan and 0. 0125 g of hydrochlorothiazide were weighed accurately and transferred quantitatively to the same flask which was half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature, and the volume was completed to the mark with the same solvent. Subsequent dilutions were made with mobile phase to achieve same concentration of the standard.

2.4.1.8 Accuracy

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 6.25µg/ml solutions for hydrochlorothiazide and 40µg/ml solutions of valsartan. This solution was injected six times.

(b) Samples

Seven 100-ml volumetric flasks were labeled, a placebo equivalent to tablet's weight was transferred to each flask. A volume of standard stock solution required to produce 40%, 60%, 80%, 100%, 120%, 140% and 160% tablet's content of both hydrochlorothiazide and valsartan was added each to different flask. The flask was half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature and completed to the mark with the same solvent. Subsequent dilutions with mobile phase similar to those made for standard preparation. Each solution was injected three times. The results were collected and subjected to statistical treatments.

2.4.1.9 Precision

(a) Standard of Precision

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 6.25µg/ml solutions for hydrochlorothiazide and 40µg/ml solutions of valsartan. This solution was injected six times.

(b) samples of Precision

Three 100-ml volumetric flasks were labled, a placebo equivalent to tablet's weight was transferred to each flask. A volume of standard stock solution required to produce 80%, 100% and 120% tablet's content of both hydrochlorothiazide and valsartan was added each to a different flask. The flask was half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature and completed to the mark with the same solvent. Subsequent dilutions were made with mobile phase similar to those made for standard preparation.

2.4.1.10 Robustness

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 6.25µg/ml solutions for hydrochlorothiazide and 40µg/ml solutions of valsartan. This solution was injected six times at each different condition.

(b) samples

A placebo equivalent to one tablet's weight was transferred to 100-ml volumetric flask, the volume required to prepare 6.25µg/ml solutions for hydrochlorothiazide and 40µg/ml solutions of valsartan was transferred quantitatively from standard stock solution to the placebo flask which was half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature and the volume was completed to the mark with the same solvent. The standard was injected six times and the sample was injected three times at each of the following conditions relative to that of the optimum condition: five degres more temperature, five degres less temperature, 5% more organic solvent in mobile phase, 5% less organic solvent in mobile phase, 5% more flow rate of mobile phase, 5% less flow rate of mobile phase, 3nm above the detection wavelength and 3nm below the detection wavelength. The results were collected and subjected to statistical treatments.

2.4.1.11 Assay of real samples

(a) Standard preparation

Subsequent dilutions from the standard stock solution were made with mobile phase to give the concentration of 40 µg/ml solutions for valsartan and 6.25 µg/ml solutions for hydrochlorothiazide. This solution was injected six times.

(b) Assay preparation

Twenty tablets weighed, transferred to a mortar and grinded. Average weight of tablet was transferred to 100-ml volumetric flask which was half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature and the volume was completed to the mark with the same solvent, Subsequent dilutions were made with mobile phase similar to those made for standard preparation to achieve target concentration.

2.4.2 Amlodipine besylate & Losartan potassium

2.4.2.1 Optimized chromatographic conditions

Phenyl hexyle column (150mm × 4.6 mm, 3 µm), and simple isocratic elusion were used(one pump required) with flow-rate of 0.8 ml/min, both active ingredients were detected at 260 nm, injection volume was 20µl (universal loop) and analysis temperature was 25^{o}C (ambient temperature).

2.4.2.2 Buffer (1% v/v formic acid)

1000-ml volumetric flask was half filled with deionised water; 10 ml of formic acid was added to the flask; the volume was completed to the mark with deionised water.

2.4.2.3 Mobile phase

- Mixture of acetonitrile and buffer was prepared in 60:40 ratio. The mixture was shaken, filtered with vacuum filtration pump through 0.45µm nylon membrane filter, and transferred to solvent reservoir and sonicated for 5 min.

2.4.2.4 Standard Stock Solution

0.05g amlodipine besylate and 0.5g losartan potassium were weighed accurately and transferred quantitatively to the same 50-ml volumetric flask. The flask was half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature, and the volume was completed to the mark with the same solvent.

2.4.2.5 System Suitability

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 20µg/ml amlodipine besylate and 200µg/ml losartan potassium. System suitability solution was injected six times.

2.4.2.6 Linearity, LOD and LOQ

Subsequent dilutions were made from the stock solution with mobile phase to give a concentrations of 8, 12, 16, 20,24 ,28 and 32 µg/ml amlodipine besylate solutions and 80, 120, 160, 200,240 ,280 and 320 µg/ml losartan potassium solutions. Each solution was injected three times and results were collected and treated to calculate LOD and LOQ from the linear regression analysis.

2.4.2.7 Specificity

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 20µg/ml amlodipine besylate and 200µg/ml losartan potassium. This solution was injected six times.

(b) Placebo

A placebo equivalent to avarege weight of one tablet was transferred to 50-ml volumetric flask, the flask was half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature, and the volume was completed to the mark with the same solvent. Subsequent dilutions were made in mobile phase with similar to htose made for standard preparation.

(c) sample

Amount of placebo equivalent to that of one tablet was transferred to 50-ml volumetric flask, the volume required to prepare 20µg/ml amlodipine besylate and 200µg/ml losartan potassium was added from standard stock solution to the flask which then half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature, and the volume was completed to the mark with the same solvent. Subsequent dilutions were made with mobile phase to achieve same concentration of the standard.

2.4.2.8 Accuracy

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 20µg/ml amlodipine besylate and 200µg/ml losartan potassium. This solution was injected six times.

(b) samples

Seven 50ml volumetric flasks were labeled; a placebo equivalent to tablet's weight was transferred to each flask. A volume of standard stock solution required to produce 40%, 60%, 80%, 100%, 120%, 140% and 160% tablet's content of both amlodipine besylate and losartan potassium was added each to different flask. The flask was half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature and completed to the mark with the same solvent. Subsequent dilutions were made with mobile phase similar to those made for standard preparation. Each solution was injected three times.

2.4.2.9 Precision

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 20µg/ml amlodipine besylate and 200µg/ml losartan potassium. This solution was injected six times.

(b) Samples

Three 50 ml volumetric flasks were labeled, a placebo equivalent to tablet's weight was transferred to each flask. A volumes of standard stock solution required to produce 80%, 100% and 120% tablet's content of both amlodipine besylate and losartan potassium were each added to different flask. The flask was half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature, and completed to the mark with the same solvent. Subsequent dilutions were made with mobile phase similar to those made for the standard preparation. For each trial the three solutions were injected three times.

2.4.2.10 Robustness

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 20µg/ml amlodipine besylate and 200µg/ml losartan potassium. This solution was injected six times at each different condition.

(b) samples

A placebo equivalent to one tablet's weight was transferred to 50-ml volumetric flask, the volume required to prepare 20µg/ml amlodipine besylate and 200µg/ml losartan potassium was transferred quantitatively from standard stock solution to the placebo flask which half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature and the volume was completed to the mark with the same solvent. Subsequent dilutions were made similar to those made for standard preparation. The standard was injected six times and the sample was injected three times at each of the following conditions relative to that of the optimum condition: five degres more temperature, five degres less temperature, 5% more organic solvent in mobile phase, 5% less organic solvent in mobile phase, 5% more flow rate of mobile phase, 5% less flow rate of mobile phase, 3nm above the detection

wavelength and 3nm below the detection wavelength. The results were collected and subjected to statistical treatments

2.4.2.11 Assay of real samples

(a) Standard preparation

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 20µg/ml amlodipine besylate and 200µg/ml losartan potassium. This solution was injected six times.

(b) Assay preparation

Twenty tablets weighed, transferred to a mortar and grinded. Average weight of tablet was transferred to 50-ml volumetric flask which was half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature, and the volume was completed to the mark with the same solvent, Subsequent dilutions were made with mobile phase similar to those made for the standard to achieve target concentration.

2.4.3 Amlodipine besylate & Atorvastatine Calcium

2.4.3.1 Optimized chromatographic conditions

Neucleodur polaratic - (50mmx2mm I.D, 1.8 µm) column and simple simple isocratic elusion were used (one pump required) with flow-rate of 0.3 ml/min, both active ingredients were detected at 240 nm, injection volume was 20µl (universal loop), and analysis temperature was 25^{o}C (ambient temperature).

2.4.3.2 Buffer (1% v/v formic acid)

1000-ml volumetric flask was half filled with deionised water, 10 ml of formic acid was added to the flask, and the volume was completed to the mark with deionised water.

2.4.3.3 Mobile phase

- Mixture of acetonitrile and buffer was prepared in 60:40 ratio respectively. the mixture was shaken, filtered with vacuum filtration pump through 0.45 nylon membrane filter, transferred to solvent reservoir and sonicated for 5 min.

2.4.3.4 Standard Stock Solution

0.1g amlodipine besylate and 0.1g atorvastatine calcium were weighed accurately and transferred quantitatively to the same 100-ml volumetric flask which was half filled with

mobile phase and sonicated for 10 minutes, cooled to room temperature, and the volume was completed to the mark with the same solvent.

2.4.3.5 System Suitability

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 20µg/ml of both active ingredients. System suitability solution was injected six times.

2.4.3.6 Linearity, LOD and LOQ

Subsequent dilutions were made from the stock solution with mobile phase to obtain a concentrations of 8, 12, 16, 20,24 ,28 and 32 µg/ml of both amlodipine besylate and Atorvastatine Calcium. Each solution was injected three times and results was collected and treatedto calculate LOD and LOQ from the linear regression.

2.4.3.7 Specificity

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 20µg/ml for both amlodipine besylate and atorvastatine calcium. This solution was injected six times.

(b) Placebo

A placebo equivalent to average weight of tablet was transferred to 100ml volumetric flask. The flask half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature, the volume was completed to the mark with the same solvent, Subsequent dilutions were made in mobile phase in same manner of standard preparation.

(c) Sample

Amount of placebo equivalent to that of one tablet was transferred to 100-ml volumetric flask, the volume required to prepare 100µg/ml of both amlodipine besylate and atorvastatine calcium was added from standard stock solution to the flask which was half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature, and the volume was completed to the mark with the same solvent. Subsequent dilutions were made in mobile phase to achieve the target concentration.

2.4.3.8 Accuracy

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 20µg/ml for both amlodipine besylate and atorvastatine calcium. This solution was injected six times.

(b) samples

Seven 100-ml volumetric flasks were labeled; a placebo equivalent to tablet's weight was transferred to each flask. A volume of standard stock solution required to produce 40%, 60%, 80%, 100%, 120%, 140% and 160% tablet's content of both amlodipine besylate and atorvastatine calcium were each added to different flask. The flask was half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature and completed to the mark with the same solvent. Subsequent dilutions were made with mobile phase similar to those made for the standard preparation. Each solution was injected three times.

- The results was collected and subjected to statistical treatments.

2.4.3.9 Precision

(a) Precision Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 20µg/ml for both amlodipine besylate and atorvastatine calcium. This solution was injected six times.

(b) Precision samples

Three 100-ml volumetric flasks were labeled; a placebo equivalent to tablet's weight was transferred to each flask. A volumes of standard stock solution required to produce 80%, 100% and 120% tablet's content of both amlodipine besylate and Atorvastatine calcium were each added to different flask. The flask was half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature, and completed to the mark with the same solvent. Subsequent dilutions were made with mobile phase similar to those made for the standard preparation. Each solution was injected three times. The results was collected and subjected to statistical treatments.

2.4.3.10 Robustness

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 20µg/ml for both amlodipine besylate and atorvastatine calcium. This solution was injected six times at each different condition.

(b) samples

A placebo equivalent to one tablet's weight was transferred to 100-ml volumetric flask. The volume required to prepare 100µg/ml for both amlodipine besylate and atorvastatine calcium was transferred quantitatively from standard stock solution to the placebo flask which was then half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature, and the volume was completed to the mark with the same solvent. Subsequent dilutions were made similar to those made for the of standard preparation. The standard was injected six times and the sample was injected three times at each of the different conditions relative to the optimum condition: five degres more temperature, five degres less temperature, 5% more organic solvent in mobile phase, 5% less organic solvent in mobile phase, 5% more flow rate of mobile phase and 5% less flow rate of mobile phase. The results were collected and subjected to statistical treatments

2.4.3.11 Assay of real samples

(a) Standard preparation

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 20µg/ml for both amlodipine besylate and Atorvastatine calcium. This solution was injected six times.

(b) Assay preparation

Twenty tablets were weighed, transferred to a mortar and grinded. Average weight of tablet was transferred to 100-ml volumetric flask which was then half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature, and then the volume was completed to the mark with the same solvent, Subsequent dilutions were made with mobile phase similar to those nade for the standard to achieve target concentration.

2.4.4 Hydrochlorothiazide, Amlodipine besylate and losartan potassium

2.4.4.1 Optimized chromatographic conditions

Phenyl hexyle column (150mm × 4.6 mm, 3 µm) and simple isocratic elusion were used (one pump required) with flow-rate of 0.8 ml/min, the three active ingredients were detected at the same wavelength 260 nm, injection volume was 20µl (universal loop) and analysis temperature was 25^{o}C (ambient temperature).

2.4.4.2 Buffer (1% v/v formic acid)

1000-ml volumetric flask was half filled with deionised water, 10 ml of formic acid was added to the flask; the volume was completed to the mark with deionised water.

2.4.3 Mobile phase

1:1 Mixture of acetonitrile : buffer was prepared as mobile phase; the mixture was shaken, filtered with vacuum filtration pump through 0.45µm nylon membrane filter, transferred to solvent reservoir and sonicated for 5 min.

2.4.4.4 Standard Stock Solution

0.125g, 0.05g and 0.5g of hydrochlorothiazide, amlodipine besylate and losartan potassium, respectively, were weighed accurately and transferred quantitatively to the same 100-ml volumetric flask. The flask was half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature, and the volume was completed to the mark with the same solvent.

2.4.4.5 System Suitability

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 25µg/ml, 10µg/ml and 100µg/ml of hydrochlorothiazide, amlodipine besylate and losartan potassium, respectively. System suitability solution was injected six times.

2.4.4.6 Linearity, LOD and LOQ

Mixed solution was prepared by subsequent dilutions from the stock solution with mobile phase to obtain a concentrations of 10, 15, 20, 25,30 ,35 and 40 µg/ml hydrochlorothiazide, 4, 6, 8, 10,12 ,14 and 16 µg/ml amlodipine besylate, and 40, 60, 80, 100,120 ,140 and 160

µg/ml losartan potassium. Each solution was injected three times and results were collected and treated to calculate LOD and LOQ from the linear regression analysis.

2.4.4.7 Specificity

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 25µg/ml, 10µg/ml and 100µg/ml of hydrochlorothiazide, amlodipine besylate and losartan potassium, respectively. This solution was injected six times.

(b) Placebo

A placebo equivalent to avarege weight of tablet was transferred to 100-ml volumetric flask. The flask was half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature, and the volume was completed to the mark with the same solvent, Subsequent dilutions were made with mobile phase similar to those made for standard preparation.

(c) Sample

Amount of placebo equivalent to one tablet's weight was transferred to 100-ml volumetric flask, the volume required to prepare 25µg/ml, 10µg/ml and 100µg/ml of hydrochlorothiazide, amlodipine besylate and losartan potassium, respectively, was added from standard stock solution to the flask which was half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature, and the volume was completed to the mark with the same solvent. Subsequent dilutions were made in mobile phase to achieve the target concentration.

2.4.4.8 Accuracy

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 25µg/ml, 10µg/ml and 100µg/ml of hydrochlorothiazide, amlodipine besylate and losartan potassium, respectively. This solution was injected six times.

(b) Samples

Seven 100-ml volumetric flasks were labeled; a placebo equivalent to tablet's weight was transferred to each flask. A volume of standard stock solution required to produce 40%, 60%, 80%, 100%, 120%, 140% and 160% tablet's content of hydrochlorothiazide, amlodipine besylate and losartan potassium was added each to different flask. The flasks were half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature and completed to the mark with the same solvent. Subsequent dilutions were made with mobile phase similar to those made for the standard preparation. Each solution was injected three times. The results were collected and subjected to statistical treatments.

2.4.4.9 Precision

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 25µg/ml, 10µg/ml and 100µg/ml of hydrochlorothiazide, amlodipine besylate and losartan potassium, respectively. This solution was injected six times.

(b) Samples

Three 100-ml volumetric flasks were labeled; a placebo equivalent to tablet's weight was transferred each flask. A volumes of standard stock solution required to produce 80%, 100% and 120% tablet's content of hydrochlorothiazide, amlodipine besylate and losartan potassium were added each to different flask. The flasks were half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature, and completed to the mark with the same solvent. Subsequent dilutions were made with mobile phase similar to those made for the standard preparation. Each solution was injected three times. The results were collected and subjected to statistical treatments.

2.4.4.10 Robustness

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 25µg/ml, 10µg/ml and 100µg/ml of hydrochlorothiazide, amlodipine besylate and losartan potassium, respectively. This solution was injected six times at each different condition.

(b) samples

A placebo equivalent to one tablet's weight was transferred to 100-ml volumetric flask. The volume required to prepare 25µg/ml, 10µg/ml and 100µg/ml of hydrochlorothiazide, amlodipine besylate and losartan potassium, respectively was transferred quantitatively from standard stock solution to the placebo flask which was then half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature, and the volume was completed to the mark with the same solvent. Subsequent dilutions were made similar to those made for the standard preparation. The standard was injected six times and the sample was injected three times at each of the different conditions relative to optimum condition: five degres more temperature, five degres less temperature, 5% more organic solvent in mobile phase, 5% less organic solvent in mobile phase, 5% more flow rate of mobile phase, 5% less flow rate of mobile phase, 3nm above the detection wavelength and 3nm below the detection wavelength. The results were collected and subjected to statistical treatments.

2.4.4.11 Assay of real samples

(a) Standard preparation

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 25µg/ml, 10µg/ml and 100µg/ml of hydrochlorothiazide, amlodipine besylate and losartan potassium, respectively. This solution was injected six times.

(b) Assay preparation

Twenty tablets was weighed, transferred to a mortar and grinded. Average weight of tablet was transferred to 100-ml volumetric flask which was then half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature, and then the volume was completed to the mark with the same solvent. Subsequent dilutions were made with mobile phase similar to those made for the standard to achieve the target concentration.

2.4.5 Hydrochlorothiazide, Amlodipine besylate & Valsartan

2.4.5.1 Optimized chromatographic conditions

Phenyl hexyle column (150mm × 4.6 mm, 3 µm) and simple isocratic elusion were used (one pump required) with flow-rate of 0.8 ml/min, the three active ingredients were detected at the same wavelength 260 nm, injection volume was 20µl (universal loop), and analysis temperature was 25^{o}C (ambient temperature).

2.4.5 .2 Buffer (1% v/v formic acid)

1000-ml volumetric flask was half filled with deionised water, 10 ml of formic acid was added to the flask, the volume was completed to the mark with deionised water.

2.4.5 .3 Mobile phase

1:1 Mixture of acetonitrile : buffer was prepared as mobile phase. the mixture was shaken, filtered with vacuum filtration pump through 0.45µm nylon membrane filter, and transferred to solvent reservoir and sonicated for 5 min.

2.4.5.4 Standard Stock Solution

0.125g, 0.05g and 1.6g of hydrochlorothiazide, amlodipine besylate and valsartan, respectively were weighed accurately and transferred quantitatively to the same 100-ml volumetric flask. The flask was half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature, and the volume was completed to the mark with the same solvent.

2.4.5.5 System Suitability

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 12.5µg/ml, 5µg/ml and 160µg/ml of hydrochlorothiazide, amlodipine besylate and valsartan, respectively. System suitability solution was injected six times.

2.4.5.6 Linearity, LOD and LOQ

Mixed solution was prepared by subsequent dilutions from the stock solution with mobile phase to obtain a concentrations of 5, 7.5, 10, 12.5,15 ,17.5 and 20 µg/ml hydrochlorothiazide, 2, 3, 5, 5,6 ,7 and 8 µg/ml amlodipine besylate, and 64, 96, 128, 160,192 ,224 and 256 µg/ml valsartan. Each solution was injected three times and results

were collected and treated. LOD and LOQ were calculated from the linear regression analysis according to ICH guidelines.

2.4.5.7 Specificity

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 12.5µg/ml, 5µg/ml and 160µg/ml of hydrochlorothiazide, amlodipine besylate and valsartan, respectively. This solution was injected six times.

(b) Placebo

A placebo equivalent to average weight of tablet was transferred to 100-ml volumetric flask. The flask was half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature, and the volume was completed to the mark with the same solvent, Subsequent dilutions were made with mobile phase similar to those made for the standard preparation.

(c) Sample

Amount of placebo equivalent to one tablet's weight was transferred to 100-ml volumetric flask, the volume required to prepare 12.5µg/ml, 5µg/ml and 160µg/ml of hydrochlorothiazide, amlodipine besylate and valsartan, respectively, was added from standard stock solution to the flask which was half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature, and the volume was completed to the mark with the same solvent. Subsequent dilutions were made with mobile phase to achieve the target concentration.

2.4.5.8 Accuracy

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 12.5µg/ml, 5µg/ml and 160µg/ml of hydrochlorothiazide, amlodipine besylate and valsartan, respectively. This solution was injected six times.

(b) samples

Seven 100-ml volumetric flasks were labeled; a placebo equivalent to tablet's weight was transferred to each flask. A volume of standard stock solution required to produce 40%,

60%, 80%, 100%, 120%, 140% and 160% tablet's content of hydrochlorothiazide, amlodipine besylate and valsartan were added each to a different flask. The flasks were half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature and completed to the mark with the same solvent. Subsequent dilutions were made with mobile phase similar to those made for the standard preparation. Each solution was injected three times. The results were collected and subjected to statistical treatments.

2.4.5.9 Precision

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 12.5µg/ml, 5µg/ml and 160µg/ml of hydrochlorothiazide, amlodipine besylate and valsartan, respectively. This solution was injected six times.

(b) Samples

Three 100-ml volumetric flasks were labeled; a placebo equivalent to tablet's weight was transferred to each flask. Volumes of standard stock solution required to produce 80%, 100% and 120% tablet's content of hydrochlorothiazide, amlodipine besylate and valsartan were added each to different flask. The flasks were half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature, and completed to the mark with the same solvent. Subsequent dilutions were made with mobile phase aimilar to those made for the standard preparation. Each solution was injected three times. The results were collected and subjected to statistical treatments.

2.4.5.10 Robustness

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 12.5µg/ml, 5µg/ml and 160µg/ml of hydrochlorothiazide, amlodipine besylate and valsartan, respectively. This solution was injected six times at each different condition.

(b) samples

A placebo equivalent to one tablet's weight was transferred to 100-ml volumetric flask. The volume required to prepare 12.5µg/ml, 5µg/ml and 160µg/ml of hydrochlorothiazide, amlodipine besylate and valsartan, respectively was transferred quantitatively from

standard stock solution to the placebo flask which was then half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature, and the volume was completed to the mark with the same solvent. Subsequent dilutions were made similar to those made for the standard preparation. The standard was injected six times and the sample was injected three times at each of the different conditions relative to optimum condition: five degres more temperature, five degres less temperature, 5% more organic solvent in mobile phase, 5% less organic solvent in mobile phase, 5% more flow rate of mobile phase, 5% less flow rate of mobile phase, 3nm above the detection wavelength and 3nm below the detection wavelength. The results were collected and subjected to statistical treatments

2.4.5.11 Assay of real sample

(a) Standard preparation

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 12.5µg/ml, 5µg/ml and 160µg/ml of hydrochlorothiazide, amlodipine besylate and valsartan, respectively. This solution was injected six times.

(b) Assay preparation

Twenty tablets weighed, transferred to a mortar and grinded. Average weight of tablet was transferred to 100-ml volumetric flask which was half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature, and the volume was completed to the mark with the same solvent. Subsequent dilutions were made with mobile phase similar to those made for the standard to achieve target concentration.

3.1 Hydrochlorothiazide & Valsartan

3.1.1 System Suitability

System suitability results for hydrochlorothiazide and valsartan are shown in Table 3.1 and Table 3.2 respectively.

Table 3.1 System suitability results for hydrochlorothiazide

No.	Retention time	Area	Theoretical plates	Tailing factor	Resolution
1	4.512	619051	7712	1.293	12.02
2	4.511	617597	7713	1.292	12.035
3	4.511	619530	7763	1.294	12.04
4	4.511	619240	7718	1.295	11.993
5	4.515	619382	7758	1.295	12.019
6	4.512	618804	7735	1.294	12.019
Avg	**4.512**	**618934**	**7733.166667**	**1.293833333**	**12.021**
STDEV	**0.001549193**	**702.5260138**	**22.7808399**	**0.001169045**	**0.016431677**
RSD	**0.034334959**	**0.113505804**	**0.294586175**	**0.090355161**	**0.136691429**

Table 3.2 System suitability results for valsartan

No.	Retention time	Area	Theoretical plates	Tailing factor	Resolution
1	2.302	562357	3219	1.346	12.02
2	2.301	562920	3238	1.344	12.035
3	2.302	562108	3222	1.347	12.04
4	2.301	562314	3177	1.351	11.993
5	2.301	563103	3178	1.352	12.019
6	2.301	561654	3199	1.343	12.019
Avg	**2.301333333**	**562409.3333**	**3205.5**	**1.347166667**	**12.021**
STDEV	**0.000516398**	**531.9983709**	**24.98599608**	**0.003656045**	**0.016431677**
RSD	**0.022439069**	**0.094592735**	**0.779472659**	**0.271387744**	**0.136691429**

3.1.2 Linearity, LOD and LOQ

i) Hydrochlorothiazide

Table 3.3 shows linearity results for hydrochlorothiazide which then treated by XLSTAT-2015 program to predict linearity data that shown in Table 3.4, Table 3.5, Figure 3.1 and Figure 3.2.

Table 3.3 linearity result for hydrochlorothiazide

Conc µg/ml	2.5	3.75	5.0	6.25	7.5	8.75	10
1	217830	336362	448082	563517	676137	795753	909592
2	219145	337416	449727	562404	676808	794042	907348
3	218759	336986	449096	562188	679858	794603	906955
avg	**218578**	**336921**	**448968.3**	**562703**	**677601**	**794799.3**	**907965**
STDEV	**675.9268**	**529.9673**	**829.89778**	**713.169685**	**1983.20372**	**872.23**	**1422.65**
RSD	**0.309238**	**0.157297**	**0.1848455**	**0.12673998**	**0.29268016**	**0.10974**	**0.15668**

Figure 3.1 shows the plot of average area versus concentrations for hydrochlorothiazide in µg/ml, the linear regression equation:

$$\text{Area} = -9735.87+91787.13*\mu g/ml$$

According to ICH guidelines, acceptance criteria is $R^2 \geq 0.997$.

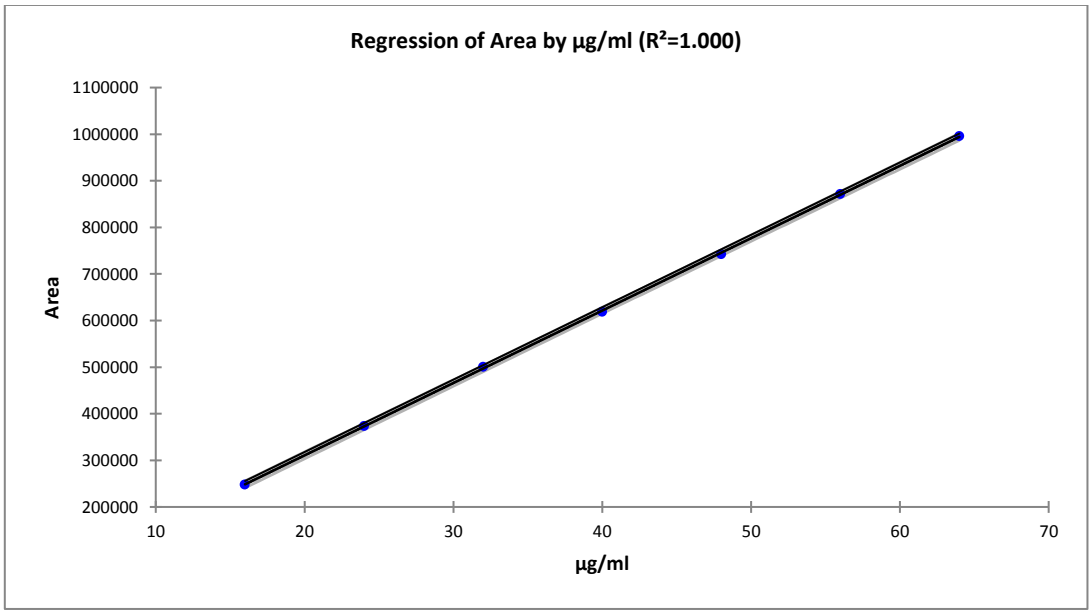

Figure 3.1 XL- STAT 2015 Graph of conc. in µg/ml Vs average area of hydrochlorothiazide

Table 3.4 XL- STAT 2015 Goodness of fit statistics for hydrochlorothiazide

Observations	7.000
Sum of weights	7.000
R^2	1.000
Adjusted R^2	1.000
MSE	2409935.580
RMSE	1552.397

Table 3.5 XL STAT 2015 predicted area for hydrochlorothiazide

Observation	Weight	µg/ml	Area	Pred. (Area)
Obs1	1	2.500	218578.000	219731.964
Obs2	1	3.750	336921.333	334465.881
Obs3	1	5.000	448968.333	449199.798
Obs4	1	6.250	562703.000	563933.714
Obs5	1	7.500	677601.000	678667.631
Obs6	1	8.750	794799.333	793401.548
Obs7	1	10.000	907965.000	908135.464

Figure 3.2 is the a plot of average area versus predicted area for hydrochlorothiazide , i.e. concentration Vs predicted concentration of hydrochlorothiazide, acceptance limit for this graph is that slope ≥ 0.997

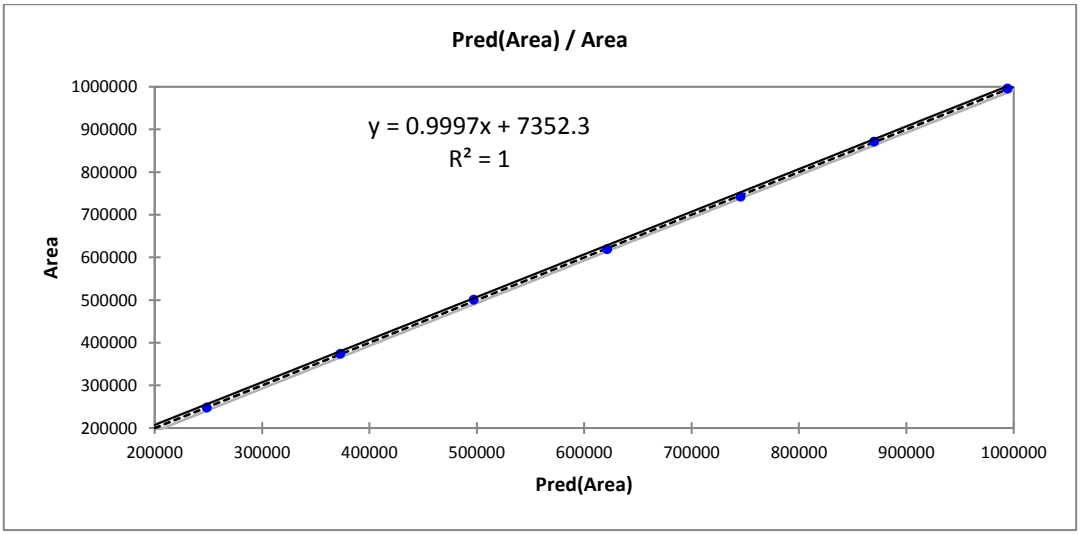

Figure 3.2 XL- STAT 2015 Graph of (area) Vs (Predicted area) for hydrochlorothiazide

Limit of detection and limit of quantitation

LOD = 3.3* (SD/S).

LOD = 3.3* (1552/91787) =

LOD = **0.056 µg/ml**

Percentage =0.056*100/6.25 = 0. 9%

LOQ = 10 * (SD/S).

LOQ = 10* (1552/91787)

LOQ = **0.17 µg/ml**

Percentage =0.17*100/6.25 = 2.7%

ii) Valsartan

Table 3.6 shows linearity results for valsartan which then treated by XLSTAT-2015 program to predict linearity data that shown in Table 3.7, Table 3.8, Figure 3.3 and Figure 3.4.

Table 3.6 linearity result for valsartan

Conc µg/ml	40	60	80	100	120	140	160
1	217830	336362	448082	563517	676137	795753	909592
2	219145	337416	449727	562404	676808	794042	907348
3	218759	336986	449096	562188	679858	794603	906955
avg	**218578**	**336921**	**448968.3**	**562703**	**677601**	**794799.3**	**907965**
STDEV	**675.9268**	**529.9673**	**829.89778**	**713.169685**	**1983.20372**	**872.232958**	**1422.65913**
RSD	**0.309238**	**0.157297**	**0.1848455**	**0.12673998**	**0.29268016**	**0.10974254**	**0.156686561**

Figure 3.3 shows the plot of average area versus concentrations for valsartan in µg/ml, the linear regression equation:

Area = -9735.87+91787.13*µg/ml

According to ICH guidelines, acceptance criteria is $R^2 \geq 0.997$.

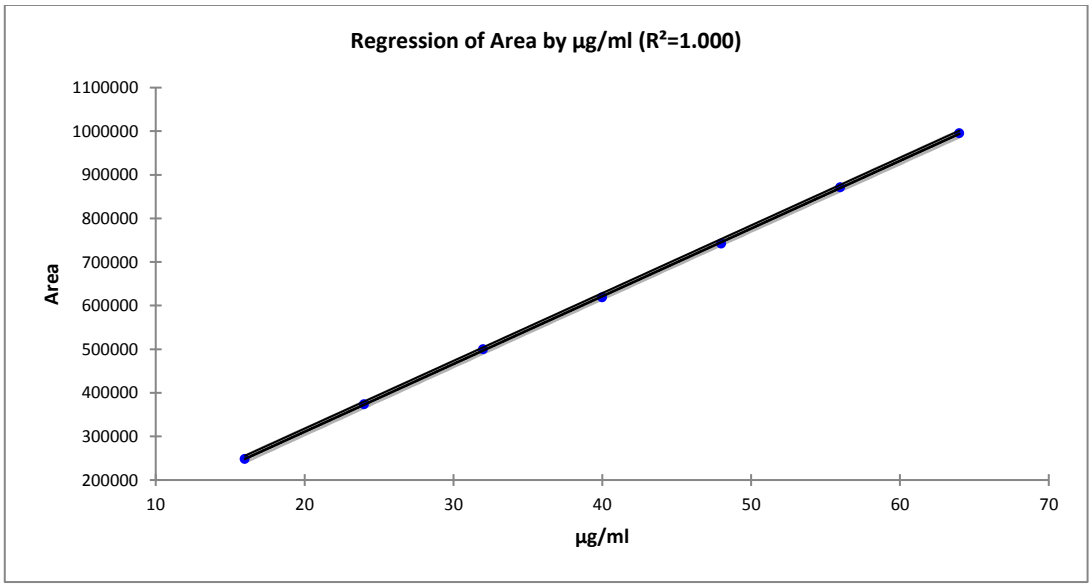

Figure 3.3 XL- STAT 2015 Graph of conc. in µg/ml Vs average area of valsartan

Linear regression equation for valsartan Area = 19.0833+15534.0551*µg/ml

Table 3.7 XL- STAT 2015 Goodness of fit statistics of Valsartan

Observations	7.000
Sum of weights	7.000
R^2	1.000
Adjusted R^2	1.000
MSE	6168027.421
RMSE	2483.551

Table 3.8 XL- STAT 2015 predicted area for Valsartan

Observation	Weight	µg/ml	Area	Predicted (Area)
Obs1	1	16.000	247967.667	248563.964
Obs2	1	24.000	373702.000	372836.405
Obs3	1	32.000	500125.667	497108.845
Obs4	1	40.000	618877.333	621381.286
Obs5	1	48.000	742322.000	745653.726
Obs6	1	56.000	871284.000	869926.167
Obs7	1	64.000	995390.333	994198.607

Figure 3.4 is the plot of average area versus predicted area for valsartan , i.e. concentration versus predicted concentration of valsartan, acceptance limit for this graph is that slope ≥ 0.997

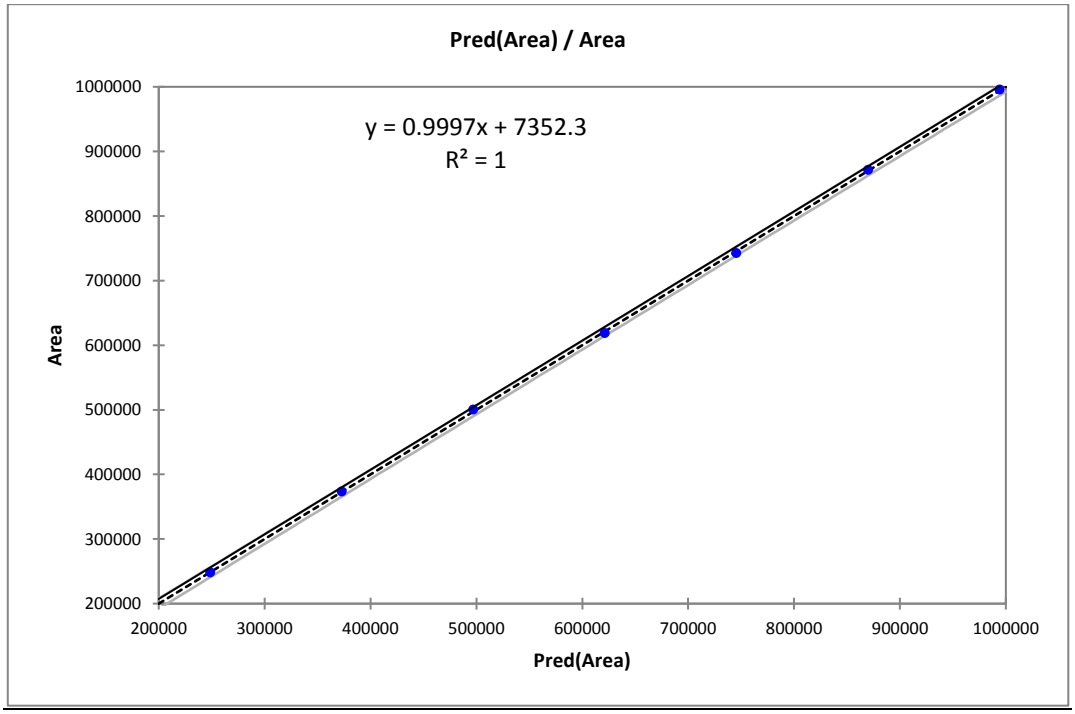

Figure 3.4 XL- STAT 2015 Graph of (area) versus (Predicted area) for valsartan

Limit of detection and limit of quantitation

LOD = 3.3* (SD/S).

LOD = 3.3* (2483.551/15534.1) =

LOD = **0.5276 µg/ml**

Percentage =0.527*100/40 = 1.3%

LOQ = 10 * (SD/S).

LOQ = 10* (2483.551/15534.1)

LOQ = **1.599 µg/ml**

Percentage =1.599*100/40 = 4%

3.1.3 Specificity

Figure 3.5 Figure 3.6 and Figure 3.7 shows the specificity chromatograms for placebo, sample and standard respectively for hydrochlorothiazide and valsartan.

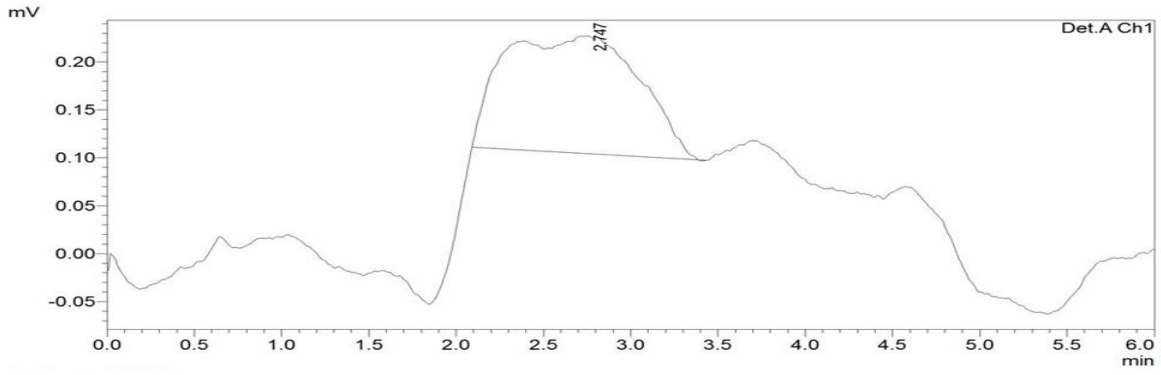

Figure 3.5 chromatogram for the Placebo of hydrochlorothiazide and valsartan

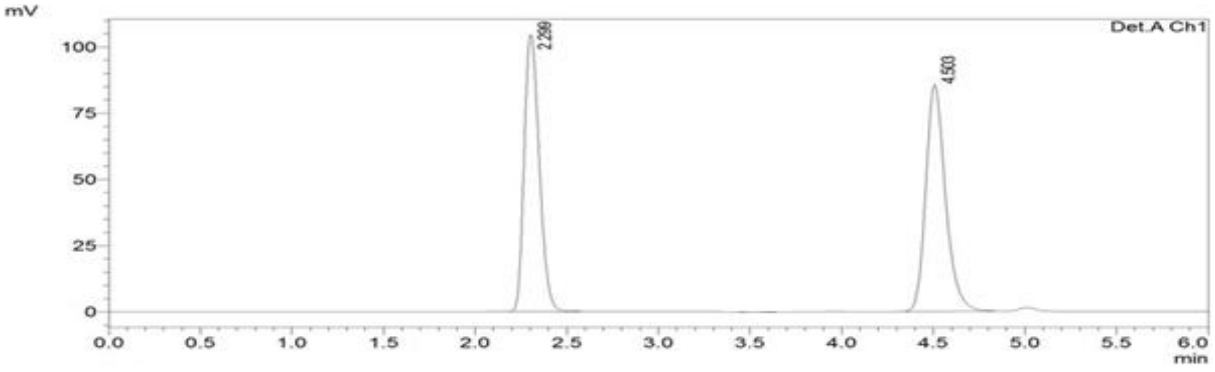

figure 3.6 chromatogram for the sample of hydrochlorothiazide and valsartan

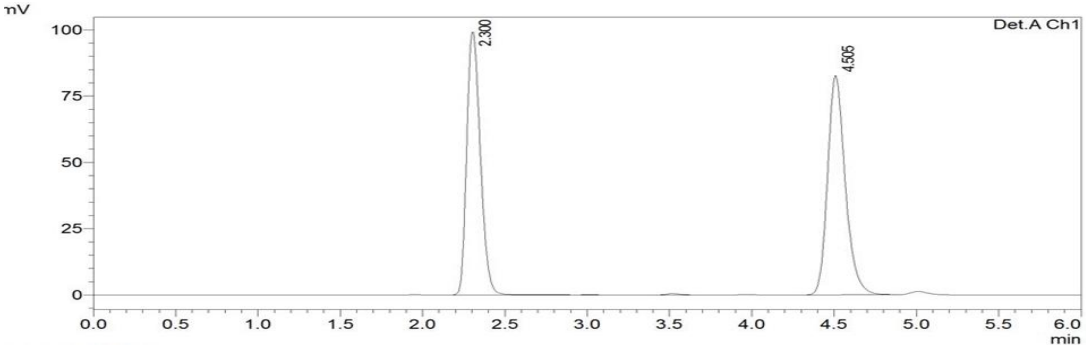

figure 3.7 chromatogram for mixed standard of hydrochlorothiazide and valsartan

3.1.4 Accuracy

Table 3.9 shows the results of mixed standard of hydrochlorothiazide and valsartan, while the accuracy results for hydrochlorothiazide and valsartan samples were shown in Table 3.10 and Table 3.11, respectively; summary of accuracy results for both components is shown in Table 3.12.

Table 3.9 Results of hydrochlorothiazide and valsartan standard for accuracy test

No.	HCTZ	Val
STD1	562357	619051
SDT2	562920	617597
STD3	562108	619530
STD4	562314	619240
STD5	563103	619382
STD6	561654	618804
Avg	**562409.3**	**618934**
STDEV	**531.9984**	**702.526**
RSD	**0.094593**	**0.113506**

Table 3.10 Accuracy results for hydrochlorothiazide

Content	40	60	80	100	120	140	160
1	226870	338202	451051	566525	667379	793744	905286
2	224239	336859	448184	565839	676732	790010	907972
3	224733	336698	449130	567195	677903	796082	903699
avg	**225555**	**337253**	**449455**	**566520**	**7E+05**	**793279**	**905652**
STDEV	1860.4	825.791	1460.87	678.02	5767.8	3062.629	2159.93
RSD	0.82481	0.24486	0.32503	0.1197	0.8557	0.386072	0.23849
RECOVERY	**40.159**	**60.0464**	**80.023**	**100.87**	**120**	**141.24**	**161.25**
RECOVERY %	**100.4**	**100.077**	**100.03**	**100.87**	**100**	**100.886**	**100.78**

Table 3.11 Accuracy results for valsartan

Content	40	60	80	100	120	140	160
1	249437	371885	496790	622595	741778	871491	989488
2	248376	370508	496878	617118	739688	868477	989034
3	248715	373768	499736	621580	742831	874099	990299
avg	**248842.67**	**372053.66**	**497801.33**	**620431**	**741432.33**	**871355.67**	**989607**
STDEV	541.898822	1636.53	1676.04	2913.68032	1599.75821	2813.44226	640.840854
RSD	0.21776765	0.439864	0.33669	0.46962198	0.21576591	0.32288104	0.06475711
RECOVERY	**40.213487**	**60.12463**	**80.445**	**100.26293**	**119.81699**	**140.81287**	**159.92253**
RECOVERY %	**100.53372**	**100.2077**	**100.557**	**100.26293**	**99.847493**	**100.58062**	**99.95158**

Table 3.12 Summary of accuracy results for hydrochlorothiazide and valsartan

Content%	HCTZ RECOVERY %	VAL RECOVERY%
40	100.3974422	100.5337177
60	100.0773311	100.2077305
80	100.029333	100.55715
100	100.8663103	100.2629265
120	100.9486576	99.84749255
140	100.8855308	100.5806208
160	100.7796096	99.95157998
avg	100.444	**100.2140567**
STDEV	2.873064524	0.518399963
RSD	2.791630046	0.517292663

3.1.5 Precision

i) Intraday Precision

Table 3.13 shows results of hydrochlorothiazide and valsartan mixed standard for intraday precision test.

Table 3.13 hydrochlorothiazide and valsartan mixed standard for intraday precision

No.	HCTZ	Val
STD1	562357	619051
SDT2	562920	617597
STD3	562108	619530
STD4	562314	619240
STD5	563103	619382
STD6	561654	618804
Avg	**562409.3**	**618934**
STDEV	**531.9984**	**702.526**
RSD	**0.094593**	**0.113506**

Tables numbered 3.14, 3.15 and 3.16 show intraday precision for 80%, 100% and 120% of hydrochlorothiazide, respectively, while tables numbered 3.17, 3.18 and 3.19 show intraday precision for 80%, 100% and 120% of valsartan, respectively. Table 3.20 show the summary of the previous six tables and the average and RSD of each five assays of the three concentrations for each active ingredient.

Table 3.14 Intraday results for 80% hydrochlorothiazide

	1st	2nd	3rd	4th	5th
1st trial	451438	448929	450046	449254	448762
2nd trial	452935	449628	451047	452890	450318
3rd trial	452472	449657	452995	451227	453319
AVG	452281.7	449404.7	451362.7	451123.7	450799.67
STDEV	766.4348	412.1945	1499.628	1820.201	2316.3688
RSD	0.16946	0.09172	0.332245	0.403482	0.5138355
RECOVERY	80.41859	79.90704	80.25519	80.21269	80.155083
RECOVERY %	100.5232	99.8838	100.319	100.2659	100.19385

Table 3.15 Intraday results for 100% hydrochlorothiazide

	1st	2nd	3rd	4th	5th
1st trial	564901	567122	563925	567319	564183
2nd trial	564019	568212	566573	564898	566210
3rd trial	566145	564565	567968	568739	564630
AVG	565021.7	566633	566155.3	566985.3	565007.67
STDEV	1068.124	1872.029	2053.606	1942.117	1064.9678
RSD	0.189041	0.330378	0.362728	0.342534	0.1884873
RECOVERY	100.4645	100.751	100.6661	100.8136	100.462
RECOVERY %	100.4645	100.751	100.6661	100.8136	100.462

Table 3.16 Intraday results for 120% hydrochlorothiazide

	1st	2nd	3rd	4th	5th
1st trial	676999	674234	677962	674986	678344
2nd trial	676114	675689	677474	676513	676976
3rd trial	676018	674680	675310	678367	677658
AVG	676377	674867.7	676915.3	676622	677659.33
STDEV	540.8022	745.433	1411.509	1693.133	684.00097
RSD	0.079956	0.110456	0.208521	0.250233	0.1009358
RECOVERY	120.2642	119.9958	120.3599	120.3077	120.49219
RECOVERY %	100.2202	99.99651	100.2999	100.2565	100.41016

Table 3.17 Intraday results for 80% valsartan

	1st	2nd	3rd	4th	5th
1st trial	497043	498611	495440	497669	497782
2nd trial	496168	496130	496445	497276	498042
3rd trial	499404	496115	495547	496704	497372
AVG	497538.3	496952	495810.7	497216.3	497732
STDEV	1673.9	1436.756	551.9478	485.259	337.78692
RSD	0.336436	0.289114	0.111322	0.097595	0.0678652
RECOVERY	80.38633	80.2916	80.1072	80.33431	80.417621
RECOVERY %	100.4829	100.3645	100.134	100.4179	100.52203

Table 3.18 Intraday results for 100% valsartan

	1st	2nd	3rd	4th	5th
1st trial	621928	619641	621031	624175	621999
2nd trial	622123	622226	622711	620865	624961
3rd trial	620434	622333	621978	620988	624691
AVG	**621495**	**621400**	**621906.7**	**622009.3**	**623883.67**
STDEV	924.0114	1524.278	842.2686	1876.53	1637.7428
RSD	0.148676	0.245297	0.135433	0.301688	0.2625077
RECOVERY	100.4138	100.3984	100.4803	100.4969	100.79971
RECOVERY %	100.4138	100.3984	100.4803	100.4969	100.79971

Table 3.19 Intraday results for 120% valsartan

	1st	2nd	3rd	4th	5th
1st trial	742235	743788	743653	742790	743965
2nd trial	739868	744172	743167	741942	741866
3rd trial	737300	741184	739109	742220	744231
AVG	739801	743048	741976.3	742317.3	743354
STDEV	2468.182	1625.649	2495.045	432.2977	1295.491
RSD	0.333628	0.218781	0.33627	0.058236	0.1742765
RECOVERY	119.5283	120.0529	119.8797	119.9348	120.1023
RECOVERY %	99.60688	100.0441	99.89976	99.94568	100.08525

Table 3.20 Summery of intraday precession for hydrochlorothiazide and valsartan

	Hydrochlorothiazide			Valsartan		
	80 %	100 %	120 %	80 %	100 %	120 %
1st trial	100.5232399	100.4644897	100.220154	100.4829136	100.4137759	99.60687785
2nd trial	99.88380349	100.7509951	99.99651302	100.3644977	100.398427	100.0440542
3rd trial	100.3189847	100.6660629	100.2999199	100.1339938	100.4802882	99.89976494
4th trial	100.2658651	100.8136423	100.2564562	100.4178825	100.4968758	99.94567721
5th trial	100.1938535	100.4620004	100.4101599	100.5220266	100.7997083	100.0852541
Avg	**100.2371493**	**100.6314381**	**100.2366406**	**100.3842628**	**100.517815**	**99.91632567**
STDEV	**0.232431309**	**0.162228254**	**0.152018877**	**0.152369903**	**0.163086006**	**0.188245273**
RSD	**0.231881403**	**0.16121031**	**0.151659988**	**0.151786643**	**0.162245873**	**0.188402918**

ii) Interday Precision

Table 3.21 shows results of hydrochlorothiazide and valsartan mixed standard for interday precision test.

Table 3.21 hydrochlorothiazide and valsartan mixed standard for interday precision

	Hydrochlorothiazide			Valsartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
STD1	562357	562234	562145	619051	619405	617450
SDT2	562920	561643	562202	617597	620000	616920
STD3	562108	561842	561732	619530	620738	617319
STD4	562314	562928	561346	619240	618144	618746
STD5	563103	563279	562280	619382	618572	617113
STD6	561654	561557	562129	618804	619959	618596
Avg.	**562409.3**	**562247.2**	**561972.3**	**618934**	**619469.7**	**617690.7**
STDEV	**531.9984**	**711.8841**	**361.0588**	**702.526**	**969.2006**	**781.9257**
RSD	**0.094593**	**0.126614**	**0.064249**	**0.113506**	**0.156457**	**0.126589**

Tables numbered 3.22, 3.23 and 3.24 shows intraday precision for 80%, 100% and 120% for both components, respectively.Table 3.25 shows the summary of interday precision, the average and RSD of each three assays of the three concentrations for each active ingredient.

Table 3.22 interday precision results for 80% of hydrochlorothiazide and valsartan

	Hydrochlorothiazide			Valsartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Trial 1	451438	450063	449890	497043	497705	495696
Trial 2	452935	448311	449074	496168	496957	497883
Trial 3	452472	449172	451850	499404	497667	497928
Avg	452281.7	449182	450271.3	497538.3	497443	497169
STDEV	766.4348	876.0428	1426.746	1673.9	421.317	1275.854
RSD	0.16946	0.195031	0.316864	0.336436	0.084697	0.256624
Recovery	80.41859	79.89049	80.1234	80.38633	80.30143	80.48835
Recovery%	100.5232	99.86311	100.1542	100.4829	100.3768	100.6104

Table 3.23 interday precision results for 100% of hydrochlorothiazide and valsartan

	Hydrochlorothiazide			Valsartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Trial 1	564901	568566	573100	621928	621502	629278
Trial 2	564019	567121	566773	622123	620721	621074
Trial 3	566145	566525	567671	620434	622595	617823
Avg	565021.7	567404	569181.3	621495	621606	622725
STDEV	1068.124	1049.518	3423.239	924.0114	941.3188	5903.271
RSD	0.189041	0.184968	0.601432	0.148676	0.151433	0.947974
Recovery	100.4645	100.8881	101.2828	100.4138	100.3449	100.815
Recovery%	100.4645	100.8881	101.2828	100.4138	100.3449	100.815

Table 3.24 interday precision for 120% of hydrochlorothiazide and valsartan

	Hydrochlorothiazide			Valsartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Trial 1	676999	677180	678987	742235	743827	743684
Trial 2	676114	677206	677792	739868	743377	743533
Trial 3	676018	678674	677255	737300	743138	743995
Avg	676377	677686.7	678011.3	739801	743447.3	743737.3
STDEV	540.8022	855.1546	886.5869	2468.182	349.8433	235.5724
RSD	0.079956	0.126187	0.130763	0.333628	0.047057	0.031674
Recovery	120.2642	120.5318	120.6485	119.5283	120.0135	120.4061
Recovery%	100.2202	100.4432	100.5404	99.60688	100.0113	100.3384

Table 3.25 interday precision summery for both hydrochlorothiazide and valsartan

	Hydrochlorothiazide			Valsartan		
	80%	100%	120%	80%	100%	120%
Trial 1	100.5232	100.4645	100.2202	100.4829	100.4138	99.60688
Trial 2	99.86311	100.8881	100.4432	100.3768	100.3449	100.0113
Trial 3	100.1542	101.2828	100.5404	100.6104	100.815	100.3384
Avg	100.1802	100.8785	100.4013	100.49	100.5246	99.98552
STDEV	0.33083	0.409242	0.164205	0.116986	0.253903	0.366454
RSD	0.330235	0.405678	0.163549	0.116415	0.252578	0.366507

3.1.6 Robustness:

The method was examined for robustness test under nine different conditions comparing the method output under each conditions with that of the optimized conditions and with permissible limits according to ICH, lastly the variation in method output was evaluated through calculation of RSD of the nine results obtained under the different nine conditions, the results shown in the followings.

i) Optimized conditions

Standard solution was injected six times while sample solution was injected three times under optimized conditions. Results of hydrochlorothiazide and valsartan standards were shown in Table 3.26 and 3.27, respectively; results of samples for both components were shown in Table 3.28.

Table 3.26 Robustness results at optimum conditions for Hydrochlorothiazide Standards

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	2.302	562357	3219	1.346	12.02
SDT2	2.301	562920	3238	1.344	12.035
STD3	2.302	562108	3222	1.347	12.04
STD4	2.301	562314	3177	1.351	11.993
STD5	2.301	563103	3178	1.352	12.019
STD6	2.301	561654	3199	1.343	12.019
Avg	2.301333333	562409.33	3205.5	1.34716667	12.021
STDEV	0.000516398	531.99837	24.98599608	0.00365605	0.01643168
RSD	0.022439069	0.0945927	0.779472659	0.27138774	0.13669143

Table 3.27 Robustness results at optimum conditions for valsartan Standards

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	4.512	619051	7712	1.293	12.02
SDT2	4.511	617597	7713	1.292	12.035
STD3	4.511	619530	7763	1.294	12.04
STD4	4.511	619240	7718	1.295	11.993
STD5	4.515	619382	7758	1.295	12.019
STD6	4.512	618804	7735	1.294	12.019
Avg	4.512	618934	7733.166667	1.29383333	12.021
STDEV	4.512	702.52601	22.7808399	0.00116905	0.01643168
RSD	0.212546175	0.1135058	0.294586175	0.09035516	0.13669143

Table 3.28 Results of hydrochlorothiazide and valsartan sample at optimum conditions

	Hydrochlorothiazide	Valsartan
1st trial	563517	619014
2nd trial	562404	619472
3rd trial	562188	618146
Avg.	562703	**618877.333**
STDEV	713.1696853	673.4815019
RSD	0.126739983	0.1088231
Recovery %	100.0522158	99.99084447

ii) 5^{o}C more

Standard solution was injected six times while sample solution was injected three times after the column temperature was raised up five degrees celsius, Results of hydrochlorothiazide and valsartan standards are shown in Table 3.29 and 3.30, respectively; results of samples for both components are shown in Table 3.31.

Table 3.29 Results of hydrochlorothiazide standard at increased temperature

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	2.301	564128	3343	1.27	12.164
SDT2	2.3	561718	3328	1.271	12.122
STD3	2.301	564596	3330	1.27	12.153
STD4	2.302	560694	3366	1.263	12.2
STD5	2.303	561256	3339	1.264	12.163
STD6	2.303	562066	3355	1.266	12.174
Avg	**2.3018**	**562409.67**	**3343.6**	**1.2668**	**12.1624**
STDEV	0.00130384	1587.6833	16.4408029	0.0035637	0.0285885
RSD	0.056644386	0.2823001	0.49170962	0.2813156	0.2350561

Table 3.30 Results of valsartan standard at increased temperature

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	4.524	618735	7695	1.254	12.164
SDT2	4.523	618922	7624	1.256	12.122
STD3	4.524	620071	7684	1.256	12.153
STD4	4.526	619920	7732	1.252	12.2
STD5	4.527	618697	7693	1.252	12.163
STD6	4.525	619311	7708	1.252	12.174
Avg	**4.525**	**619276**	**7688.2**	**1.2536**	**12.1624**
STDEV	0.001581139	**600.20397**	40.2268567	0.0021909	0.0285885
RSD	0.034942295	**0.0969203**	0.52322854	0.1747679	0.2350561

Table 3.31 Results of hydrochlorothiazide and valsartan sample at increased temperature

No.	Hydrochlorothiazide	Valsartan
1st trial	568556	626993
2nd trial	563471	626311
3rd trial	565930	619145
Avg.	565985.6667	624149.6667
STDEV	2542.957006	4347.562229
RSD	0.449297068	0.69655765
Recovery %	100.635835	100.786994

iii) 5^{o}C less

Standard solution was injected six times while sample solution was injected three times after the column temperature was decreased five celsius degrees . Results of hydrochlorothiazide and valsartan standards are shown in Table 3.32 and 3.33, respectively; results of samples for both components are shown in Table 3.34.

Table 3.32 Results of hydrochlorothiazide standard at decreased temperature

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	2.301	561212	3269	1.265	12.145
SDT2	2.301	561054	3334	1.266	12.194
STD3	2.301	561034	5509	1.249	12.275
STD4	2.301	561583	3264	1.274	12.123
STD5	2.301	560657	3399	1.249	12.297
STD6	2.301	561887	3326	1.271	12.169
Avg	**2.301**	**561237.83**	**3766.4**	**1.2618**	**12.2116**
STDEV	0	436.79167	975.316	0.0120291	0.0729507
RSD	2.301	561212	3269	1.265	12.145

Table 3.33 Results of valsartan standard at decreased temperature

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	4.53	619672	7739	1.265	12.145
SDT2	4.529	619894	7734	1.258	12.194
STD3	4.529	620049	7871	1.254	12.275
STD4	4.532	618821	7674	1.256	12.123
STD5	4.53	619122	7834	1.252	12.297
STD6	4.528	618916	7689	1.257	12.169
Avg	**4.5296**	**619412.33**	**7760.4**	**1.2554**	**12.2116**
STDEV	0.001516575	**526.32487**	87.9050624	0.0024083	0.0729507
RSD	0.033481435	**0.0849716**	1.13273881	0.1918368	0.5973883

Table 3.34 Results of hydrochlorothiazide and valsartan sample at decreased temperature

No.	Hydrochlorothiazide	Valsartan
1st trial	567478	621315
2nd trial	562445	620176
3rd trial	566209	618126
Avg.	565377.3333	619872.3333
STDEV	2617.541658	**1616.041563**
RSD	0.462972515	**0.260705548**
Recovery %	**100.737566**	**100.074264**

iv) 5% more flow

Standard solution was injected six times while sample solution was injected three times after increasing the flow rate 5% of its optimized value. Results of hydrochlorothiazide and valsartan standards are shown in table 3.35 and 3.36, respectively; results of samples for both components are shown in Table 3.37.

Table 3.35 Results of hydrochlorothiazide standard at increased flow rate

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	2.191	543053	3144	1.361	11.805
SDT2	2.189	541657	3209	1.369	11.797
STD3	2.191	540181	3217	1.365	11.933
STD4	2.19	542953	3177	1.367	11.869
STD5	2.19	542876	3145	1.345	11.843
STD6	2.919	542443	3158	1.354	11.805
Avg	**2.3358**	**542193.83**	**3181.2**	**1.36**	**11.8494**
STDEV	0.326019478	1111.9015	31.3081459	0.010198	0.0550709
RSD	13.95750826	0.2050745	0.98416151	0.7498558	0.4647566

Table 3.36 Results of valsartan standard at increased flow rate

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	4.278	592964	7534	1.298	11.805
SDT2	4.255	591731	7567	1.294	11.797
STD3	4.299	591865	7482	1.296	11.933
STD4	4.278	591898	7587	1.292	11.869
STD5	4.277	591826	7590	1.295	11.843
STD6	4.278	592393	7501	1.295	11.805
Avg	**4.2774**	**592112.83**	**7545.4**	**1.2944**	**11.8494**
STDEV	0.015565989	**477.20495**	50.4410547	0.0015166	0.0550709
RSD	0.36391239	**0.0805936**	0.66850074	0.1171643	0.4647566

Table 3.37 Results of hydrochlorothiazide and valsartan sample at increased flow rate

No.	Hydrochlorothiazide	Valsartan
1st trial	544271	590071
2nd trial	543494	595350
3rd trial	543746	596124
Avg.	543837	593848.3333
STDEV	396.4126638	3294.078677
RSD	0.072891816	0.554700332
Recovery %	100.303059	100.293103

v) 5% less flow

Standard solution was injected six times while sample solution was injected three times after decreasing the flow rate 5% of its optimized value. Results of hydrochlorothiazide and valsartan standards are shown in Table 3.38 and 3.39, respectively; results of samples for both components are shown in Table 3.40.

Table 3.38 Results of hydrochlorothiazide standard at decreased flow rate

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	2.423	601965	3563	1.387	12.058
SDT2	2.418	601728	3289	1.338	12.131
STD3	2.416	602603	3276	1.293	12.014
STD4	2.417	603250	3260	1.343	12.119
STD5	2.418	601967	3306	1.348	12.155
STD6	2.417	603043	3338	1.347	12.2
Avg	**2.4172**	**602426**	**3293.8**	**1.3338**	**12.1238**
STDEV	0.00083666	632.67685	29.9365997	0.0231452	0.0687583
RSD	0.034612776	0.1050215	0.90887727	1.7352822	0.5671347

Table 3.39 Results of valsartan standard at decreased flow rate

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	4.756	653871	8890	1.278	12.058
SDT2	4.729	655984	7929	1.301	12.131
STD3	4.719	655934	7741	1.293	12.014
STD4	4.733	655530	7916	1.298	12.119
STD5	4.738	652968	7875	1.264	12.155
STD6	4.935	655402	7898	1.298	12.2
Avg	**4.7708**	**654948.17**	**7871.8**	**1.2908**	**12.1238**
STDEV	0.092055418	**1238.6009**	75.8795098	0.0152545	0.0687583
RSD	1.929559363	**0.1891143**	0.96394103	1.1817871	0.5671347

Table 3.40 Results of hydrochlorothiazide and valsartan sample at decreased flow rate

No.	Hydrochlorothiazide	Valsartan
1st trial	606573	657871
2nd trial	607089	653717
3rd trial	608125	656365
Avg.	607262.3333	655984.3333
STDEV	790.3855599	2103.000079
RSD	0.130155538	0.320586937
Recovery %	100.80281	100.158206

vi) 5% more organic solvent

Standard solution was injected six times while sample solution was injected three times after increasing of organic solvent in mobile phase 5% more than optimized value. Results of hydrochlorothiazide and valsartan standards are shown in Table 3.41 and 3.42, respectively; results of samples for both components are shown in Table 3.43.

Table 3.41 Results of hydrochlorothiazide standard at increased organic solvent

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	2.295	560930	2530	1.203	8.502
SDT2	2.295	561581	2495	1.197	8.398
STD3	2.295	561517	2516	1.195	8.438
STD4	2.294	560780	2504	1.194	8.432
STD5	2.295	561171	2529	1.194	8.479
STD6	**2.2948**	**561147.5**	**2514.8**	**1.1966**	**8.4498**
Avg	0.000447214	336.35978	15.3525242	0.0037815	0.040978
STDEV	0.019488129	0.0599414	0.61048689	0.3160232	0.4849587
RSD	2.295	560930	2530	1.203	8.502

Table 3.42 Results of valsartan standard at increased organic solvent

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.859	624258	6985	1.323	5.903
SDT2	3.857	622132	6958	1.325	5.88
STD3	3.841	622888	6944	1.323	5.84
STD4	3.846	624076	6944	1.326	5.835
STD5	3.844	623263	6960	1.323	5.821
STD6	3.851	623761	6974	1.322	5.824
Avg	**3.8478**	**623396.33**	**6956**	**1.3238**	**5.84**
STDEV	0.006300794	**801.51324**	12.5698051	0.0016432	0.0236749
RSD	0.163750548	**0.128572**	0.1807045	0.1241251	0.4053918

Table 3.43 Results of hydrochlorothiazide and valsartan sample at increased organic solvent

No.	Hydrochlorothiazide	Valsartan
1st trial	570959	635449
2nd trial	570638	621206
3rd trial	570643	624854
Avg.	570746.6667	627169.6667
STDEV	183.9030541	7398.479326
RSD	0.032221485	1.179661536
Recovery %	101.710632	100.605286

vii) 5% less organic solvent

Standard solution was injected six times while sample solution was injected three times after decreasing of organic solvent in mobile phase 5% more than optimized value. Results of hydrochlorothiazide and valsartan standards are shown in Table 3.44 and 3.45, respectively; results of samples for both components are shown in Table 3.46.

Table 3.44 Results of hydrochlorothiazide standard at decreased organic solvent

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	2.308	565569	3539	1.406	15.467
SDT2	2.306	565207	3645	1.392	15.648
STD3	2.308	561992	3551	1.407	15.502
STD4	2.307	567503	3529	1.413	15.713
STD5	2.301	567981	3569	1.409	15.747
STD6	2.308	568086	3524	1.397	15.791
Avg	**2.306**	**566056.33**	**3563.6**	**1.4036**	**15.6802**
STDEV	0.002915476	2340.7007	48.9366938	0.0087636	0.1124798
RSD	0.126430006	0.4135102	1.37323756	0.6243631	0.7173364

Table 3.45 Results of valsartan standard at decreased organic solvent

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	5.259	611146	8639	1.262	15.467
SDT2	5.262	611142	8746	1.252	15.648
STD3	5.265	608109	8644	1.268	15.502
STD4	5.331	608148	8635	1.252	15.713
STD5	5.324	610645	8716	1.255	15.747
STD6	5.339	609035	8742	1.254	15.791
Avg	**5.3042**	**609704.17**	**8696.6**	**1.2562**	**15.6802**
STDEV	0.037545972	**1445.3658**	53.4770979	0.0067231	0.1124798
RSD	0.707853622	**0.2370602**	0.6149196	0.535193	0.7173364

Table 3.46 Results of hydrochlorothiazide and valsartan sample at increased organic solvent

No.	Hydrochlorothiazide	Valsartan
1st trial	570644	613758
2nd trial	567613	610380
3rd trial	570404	610263
Avg.	569553.6667	611467
STDEV	1684.945202	**1984.926447**
RSD	0.295836073	**0.324617101**
Recovery %	**100.617842**	**100.289129**

viii) 3nm more

Standard solution was injected six times while sample solution was injected three times after increasing the 3nm more than the optimized detection wavelength. Results of hydrochlorothiazide and valsartan standards are shown in Table 3.47 and 3.48, respectively; results of samples for both components are shown in Table 3.49.

Table 3.47 Results of hydrochlorothiazide standard at increased wavelength detection

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	2.303	459758	3087	1.36	11.808
SDT2	2.305	462059	3180	1.357	11.923
STD3	2.304	462988	3235	1.365	12.209
STD4	2.303	461203	3232	1.365	12.148
STD5	2.303	461038	3249	1.361	12.154
STD6	2.302	459387	3218	1.358	12.04
Avg	**2.3034**	**461072.17**	**3222.8**	**1.3612**	**12.0948**
STDEV	0.001140175	1358.6289	26.3381852	0.0037683	0.1139022
RSD	0.049499671	0.2946673	0.81724541	0.2768358	0.9417448

Table 3.48 Results of valsartan standard at increased wavelength detection

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	4.518	540494	7450	1.294	11.808
SDT2	4.533	538752	7434	1.292	5.708
STD3	4.565	538690	7681	1.293	5.735
STD4	4.549	538099	7670	1.365	5.737
STD5	4.543	538812	7711	1.299	5.725
STD6	4.518	538544	7715	1.303	5.726
Avg	**4.5416**	**538898.5**	**7642.2**	**1.3104**	**5.7262**
STDEV	0.017572706	**822.54574**	117.960587	0.0308513	0.0114761
RSD	0.386927649	**0.1526346**	1.54354226	2.3543388	0.2004132

Table 3.49 Results of hydrochlorothiazide and valsartan sample at increased wavelength detection

No.	Hydrochlorothiazide	Valsartan
1st trial	463552	539881
2nd trial	464664	543285
3rd trial	463482	542205
Avg.	463899.3333	541790.3333
STDEV	663.1450319	1739.472717
RSD	0.142950202	0.321060124
Recovery %	100.613172	100.536619

ix) 3nm less

Standard solution was injected six times while sample solution was injected three times after decreasing the 3nm more than the optimized detection wavelength. Results of hydrochlorothiazide and valsartan standards are shown in Table 3.50 and 3.51, respectively; results of samples for both components are shown in Table 3.52.

Table 3.50 Results of hydrochlorothiazide standard at increased wavelength detection

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	2.302	616437	3208	1.335	12.071
SDT2	2.299	616211	3272	1.343	12.027
STD3	2.301	616902	3215	1.352	11.913
STD4	2.301	617718	3199	1.356	11.848
STD5	2.3	615886	3215	1.348	11.9
STD6	2.301	618180	3218	1.345	11.92
Avg	**2.3004**	**616889**	**3223.8**	**1.3488**	**11.9216**
STDEV	0.000894427	897.11627	27.9588984	0.0052631	0.0653246
RSD	0.038881377	0.1454259	0.86726529	0.3902045	0.5479514

Table 3.51 Results of valsartan standard at increased wavelength detection

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	4.506	740051	7903	1.322	12.071
SDT2	4.49	741396	7772	1.305	12.027
STD3	4.485	739769	7703	1.322	11.913
STD4	4.478	738765	7657	1.305	11.848
STD5	4.478	740215	7730	1.306	11.9
STD6	4.495	738926	7620	1.305	11.92
Avg	**4.4852**	**739853.67**	**7696.4**	**1.3086**	**11.9216**
STDEV	0.007463243	**959.29384**	59.7436189	0.0075033	0.0653246
RSD	0.166397112	**0.1296599**	0.77625408	0.5733863	0.5479514

Table 3.52 Results of hydrochlorothiazide and valsartan sample at increased wavelength detection

No.	Hydrochlorothiazide	Valsartan
1st trial	618253	739175
2nd trial	617732	741323
3rd trial	618871	742871
Avg.	618285.3333	741123
STDEV	570.1879807	**1856.099135**
RSD	0.092220849	**0.250444142**
Recovery %	**100.226351**	**100.171565**

Summary of recovery for both components at the nine different conditions, average and RSD are shown in Table 3.53.

Table 3.53 Hydrochlorothiazide and valsartan recovery at all robustness conditions

No	Condition	Hydrochlorothiazide	Valsartan
1	Optimized conditions	100.05222	99.990844
2	Mor 5 degree Celsius	100.63584	100.78699
3	less 5 degree Celsius	100.73757	100.07426
4	5% More flow rate	100.30306	100.2931
5	5% less flow rate	100.80281	100.15821
6	5% more Organic solvent	101.71063	100.60529
7	5% less Organic solvent	100.61784	100.28913

3.1.7 Assay:

Standard solution and sample solution were prepared as described in section (2-4-1-11); standard solution was injected six times, while sample solution was injected three times, the average of each was used for assay calculations as shown in table 3.54 and 3.55

Table 3.54 Results of mixed standard for assay

	Hydrochlorothiazide	Valsartan
1	619051	562357
2	617597	562920
3	619530	562108
4	619240	562314
5	619382	563103
6	618804	561654
Avg	618934	562409.33
STDEV	702.52601	531.99837
RSD	0.1135058	0.0945927

Table 3.55 Assay results for hydrochlorothiazide and valsartan

	Hydrochlorothiazide	Valsartan
1st trial	622226	568212
2nd trial	624691	564630
3rd trial	621031	563925
AVG	622649.3	565589
STDEV	1866.362	2298.772
RSD	0.299745	0.406439
Assay	100.6003	100.5654

3.2 Amlodipine besylate and losartan potassium

3.2.1 System Suitability

System suitability results for amlodipine besylate and losartan potassium are shown in Table 3.56 and Table 3.57, respectively.

Table 3.56 System suitability results for amlodipine besylate

No.	Retention time	Area	Theoretical plates	Tailing factor	Resolution
1	305882	3.36	5677	1.65	6.061
2	305538	3.306	5577	1.633	6.081
3	306284	3.288	5550	1.644	6.103
4	306195	3.28	5468	1.64	6.057
5	306432	3.284	5490	1.658	6.061
6	307485	3.301	5444	1.651	6.037
Avg	**306302.7**	**3.1365**	**5534.333333**	**1.646**	**6.066666667**
STDEV	**661.552**	**0.41037629**	**85.95968047**	**0.008876936**	**0.022642144**
RSD	**0.21598**	**1.0838926**	**1.553207501**	**0.539303549**	**0.373222147**

Table 3.57 System suitability results for losartan potassium

No.	Retention time	Area	Theoretical plates	Tailing factor	Resolution
1	4012771	4.46	9352	1.361	6.061
2	4018013	4.402	9198	1.36	6.081
3	4020623	4.382	9255	1.361	6.103
4	4023237	4.375	9047	1.369	6.057
5	4023730	4.381	8997	1.371	6.061
6	4023393	4.392	9065	1.371	6.037
Avg	**4020295**	**4.39866667**	**9152.333333**	**1.3655**	**6.066666667**
STDEV	**4289.006**	**4.38844444**	**138.0893431**	**0.005357238**	**0.022642144**
RSD	**0.106684**	**1.38618519**	**1.508788393**	**0.392327945**	**0.373222147**

3.2.2 Linearity, LOD and LOQ

i) amlodipine besylate

Table 3.58 show linearity results for amlodipine besylate which then treated by XLSTAT-2015 program to predict linearity data that shown in Table 3.59, Table 3.60, Figure 3.8 and Figure 3.9.

Table 3.58 linearity result for amlodipine besylate

Conc µg/ml	40	60	80	100	120	140	160
1	123504	183393	246710	306503	367389	430185	490588
2	122233	183203	246988	306273	366546	430774	492011
3	120951	183227	245386	306439	367108	430546	492888
avg	**122229**	**183274**	**246361**	**306405**	**367014.3**	**430501.7**	**491829**
STDEV	1276.5039	103.4666	856.0241	118.70973	429.23459	296.992144	1160.75105
RSD	1.0443516	0.056454	0.347467	0.03874275	0.11695309	0.06898745	0.236007037

Figure 3.8 shows the plot of average area versus concentrations for amlodipine besylate in µg/ml, the linear regression equation:

Area = -1038.33+15392.024*µg/ml

According to ICH guidelines, acceptance criteria is $R^2 \geq 0.997$.

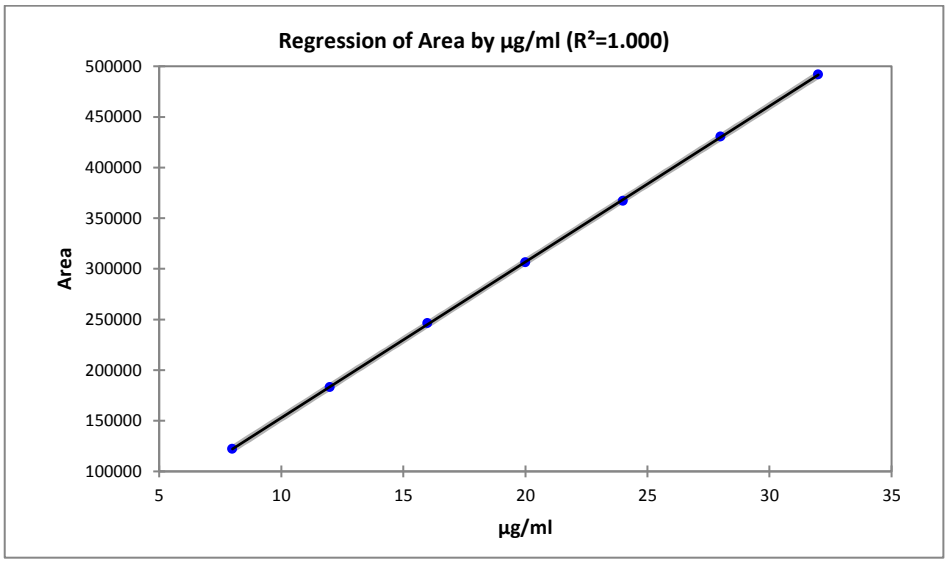

Figure 3.8 XL- STAT 2015 plot of conc. in µg/ml vs. average area of amlodipine besylate

Table 3.59 XL- STAT 2015 Goodness of fit statistics for amlodipine besylate

Goodness of fit statistics:	
Observations	7.000
Sum of weights	7.000
R^2	1.000
Adjusted R^2	1.000
MSE	771804.298
RMSE	878.524

Table 3.60 XL STAT 2015 predicted area for amlodipine besylate

Observation	Weight	µg/ml	Area	Pred(Area)
Obs1	1	8.000	122229.333	122097.857
Obs2	1	12.000	183274.333	183665.952
Obs3	1	16.000	246361.333	245234.048
Obs4	1	20.000	306405.000	306802.143
Obs5	1	24.000	367014.333	368370.238
Obs6	1	28.000	430501.667	429938.333
Obs7	1	32.000	491829.000	491506.429

Figure 3.9 is the a plot of average area versus predicted area for amlodipine , i.e. concentration Vs predicted concentration of amlodipine, acceptance limit for this graph is that slope ≥ 0.997

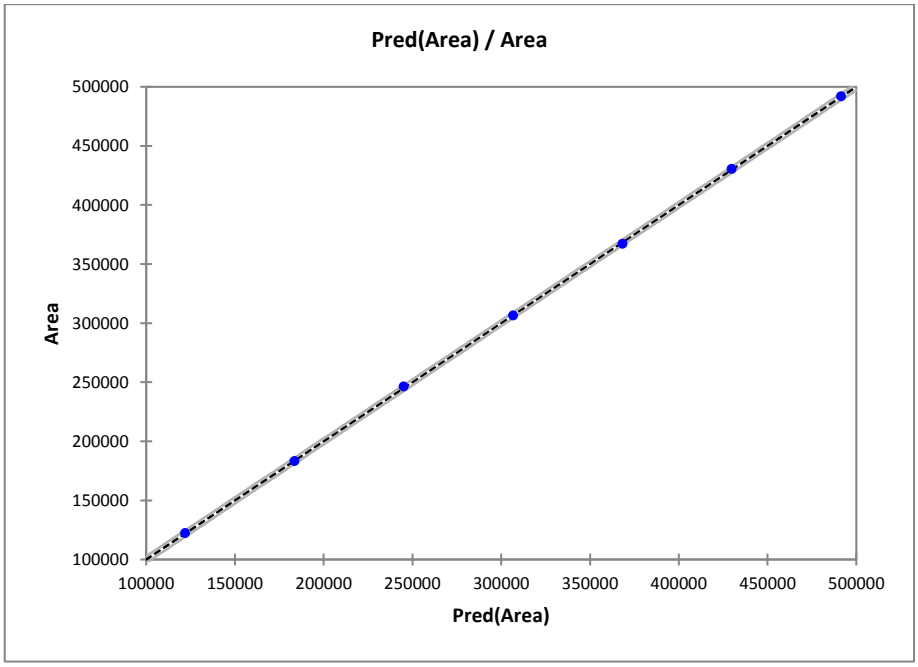

Figure 3.9 XL- STAT 2015 Graph of (area) Vs (Predicted area) for amlodipine

Limit of detection and limit of quantitation

LOD = 3.3* (SD/S).

LOD = 3.3* (878/15392) = **0.19 µg/ml**

LOD % (relative to target concentration) = 0.19*100/20 = 0.8%

LOQ = 10 * (SD/S).

LOQ = 10* (878/15392) = **0.57 µg/ml**

ii) Losartan

Table 3.61 shows linearity results for losartan potassium which then treated by XLSTAT-2015 program to predict linearity data that shown in table 3.62, table 3.63, Figure 3.10 and Figure 3.11.

Table 3.61 linearity result for losartan potassium

Conc µg/ml	40	60	80	100	120	140	160
1	1610876	2409609	3232352	4026679	4814558	5641827	6420072
2	1614711	2409515	3239641	4028065	4835309	5645921	6400630
3	1614960	2410637	3226800	4029694	4807605	5618099	6433992
avg	**1613516**	**2409920**	**3232931**	**4028146**	**4819157**	**5635282**	**6418231.33**
STDEV	2289.4061	622.4286	6440.051	1509.13121	14413.303	15021.3321	16756.99261
RSD	0.1418893	0.025828	0.199202	0.03746466	0.29908347	0.26655864	0.261084273

Figure 3.10 shows the plot of average area versus concentrations for losartan potassium in µg/ml, the linear regression equation:

$$\text{Area} = 13330.33 + 20045.62 * \mu g/ml$$

According to ICH guidelines, acceptance criteria is $R^2 \geq 0.997$.

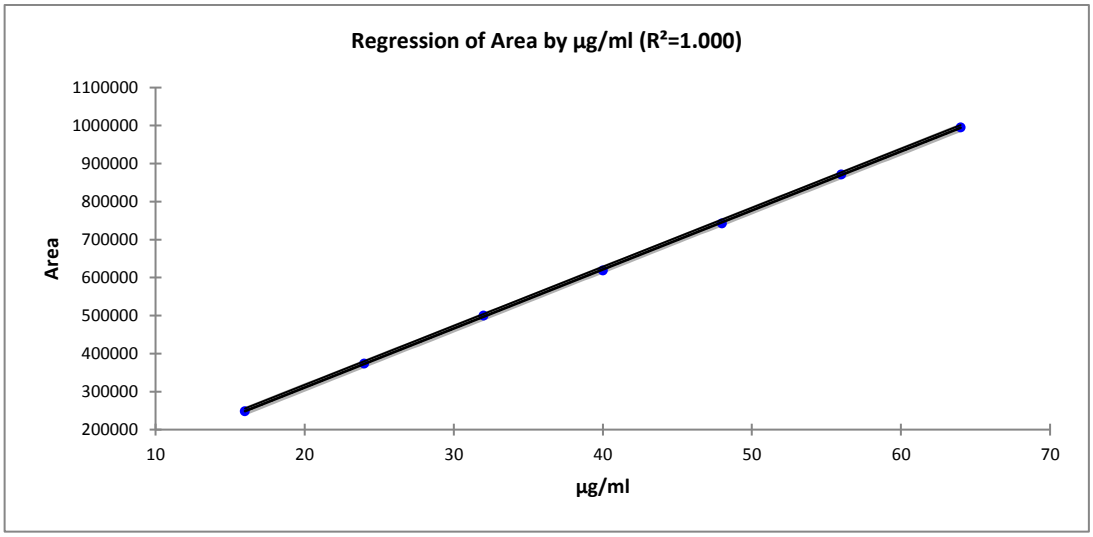

Figure 3.10 XL- STAT 2015 Graph of conc. in µg/ml Vs average area of losartan potassium

Table 3.62 XL- STAT 2015 Goodness of fit statistics of losartan potassium

Goodness of fit statistics:	
Observations	7.000
Sum of weights	7.000
R^2	1.000
Adjusted R^2	1.000
MSE	95834056.965
RMSE	9789.487

Table 3.63 XL- STAT 2015 predicted area for losartan potassium

Observation	Weight	µg/ml	Area	Pred(Area)
Obs1	1	80.000	1613515.667	1616980.143
Obs2	1	120.000	2409920.333	2418805.048
Obs3	1	160.000	3232931.000	3220629.952
Obs4	1	200.000	4028146.000	4022454.857
Obs5	1	240.000	4819157.333	4824279.762
Obs6	1	280.000	5635282.333	5626104.667
Obs7	1	320.000	6418231.333	6427929.571

Figure 3.11 is the plot of average area versus predicted area for losartan potassium , i.e. concentration Vs predicted concentration of losartan potassium, acceptance limit for this graph is that slope ≥ 0.997

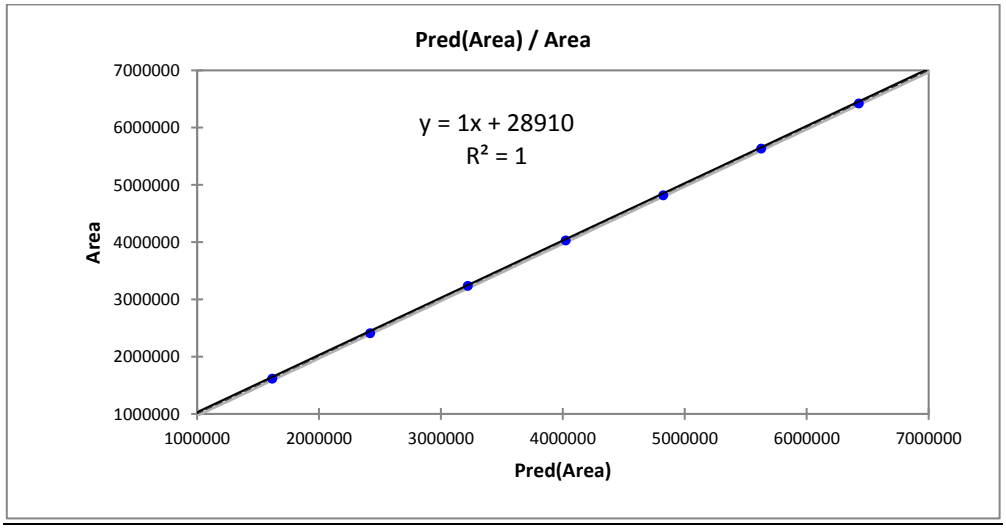

Figure 3.11 XL- STAT 2015 Graph of (area) Vs (Predicted area) for losartan potassium

Limit of Detection and Quantitation

LOD = 3.3* (SD/S).

LOD = 3.3* (9789/20045) = **1.6 µg/ml**

LOD % (relative to target concentration) = 1.6*100/200 = 0.8%

LOQ = 10 * (SD/S).

LOQ = 10* (9789/20045) = **4.8 µg/ml**

LOQ % (relative to target concentration) = 4.8*100/200 = 2.4%

3.2.3 Specificity

Figure 3.5 Figure 3.6 and Figure 3.7 shows the specificity chromatograms for placebo, sample and standard, respectively; for amlodipine besylate and losartan potassium.

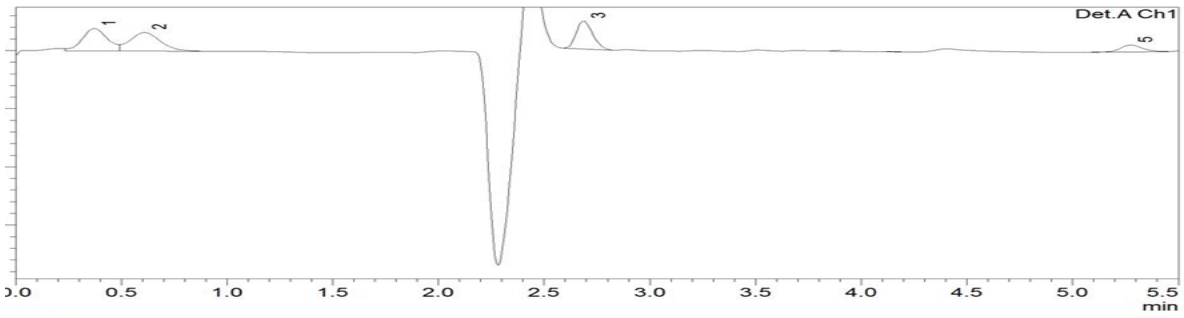

Figure 3.12 chromatogram for the Placebo of hydrochlorothiazide and losartan potassium

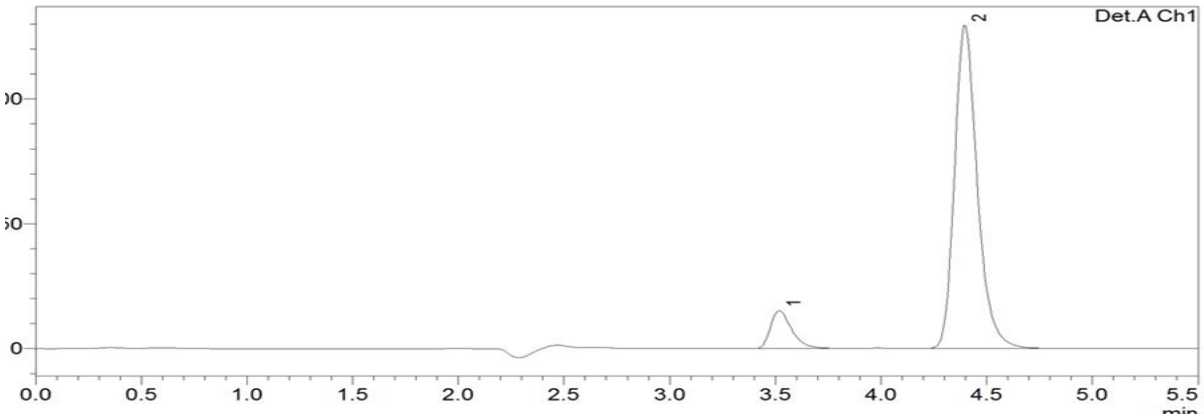

figure 3.13 chromatogram for the sample of amlodipine besylate and losartan potassium

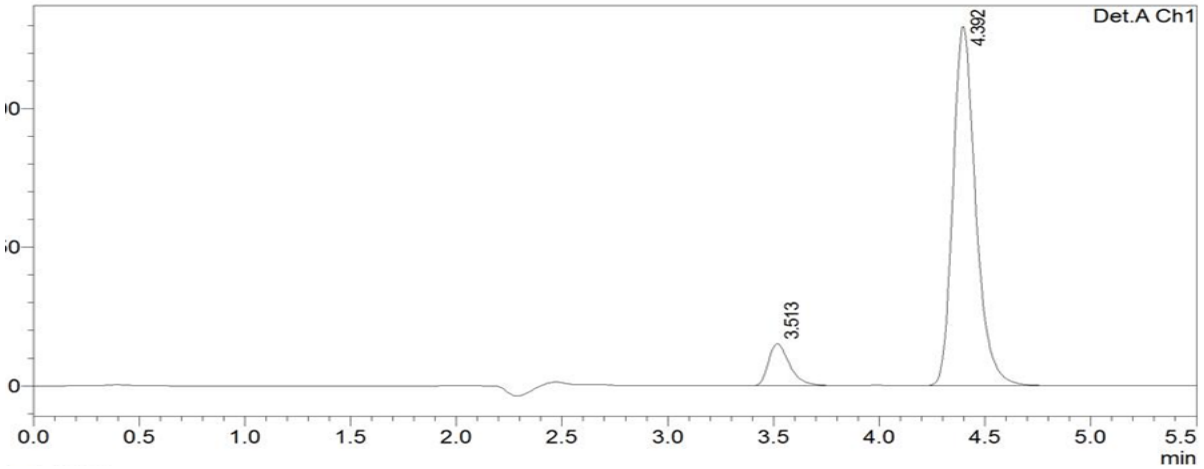

figure 3.14 chromatogram for mixed standard of amlodipine besylate and losartan potassium

3.2.4 Accuracy

Table 3.64 shows the results of mixed standard of amlodipine besylate and losartan potassium, while the accuracy results for samples are shown in Table 3.65 and Table 3.66, respectively; summary of accuracy results for both components is shown in Table 3.67.

Table 3.64 Results of amlodipine besylate and losartan standard for accuracy test

No.	HCTZ	Val
STD1	Amlodipine	Losartan
SDT2	305882	4012771
STD3	305538	4018013
STD4	306284	4020623
STD5	306195	4023237
STD6	306432	4023730
Avg	307485	4023393
STDEV	**306302.7**	**4020295**
RSD	661.55201	4289.0063

Table 3.65 Accuracy results for amlodipine

Content	40	60	80	100	120	140	160
1	122376	183133	245821	305628	367451	428743	489622
2	122420	184144	245852	305314	366546	430400	488167
3	122551	183721	245949	305289	367485	429390	488890
avg	**122449**	**183666**	**245874**	**305410**	**367161**	**429511**	**488893**
STDEV	91.032961	507.739106	66.7757441	188.9189	532.5883	835.1006	727.50464
RSD	0.0743436	0.27644698	0.02715852	0.061857	0.145056	0.194431	0.1488065
RECOVERY	**39.97647**	**59.96226**	**80.271583**	**99.7087**	**119.869**	**140.224**	**159.6111**
RECOVERY %	**99.94118**	**99.937099**	**100.33948**	**99.7087**	**99.8905**	**100.16**	**99.75693**

Table 3.66 Accuracy results for losartan

Content	40	60	80	100	120	140	160
1	1612979	2412016	3235948	4017636	4782932	5583003	6407021
2	1613082	2423934	3236321	4018188	4795053	5595200	6398694
3	1613918	2416244	3235999	4018278	4786685	5594811	6393788
avg	**1613326**	**2417398**	**3236089**	**4018034**	**4788223**	**5591005**	**6399834**
STDEV	514.97994	6042.22376	202.243254	347.6032	6205.2	6932.376	6689.7939
RSD	0.0319204	0.24994741	0.00624962	0.008651	0.129593	0.123992	0.1045307
RECOVERY	**40.12956**	**60.129874**	**80.493838**	**99.9438**	**119.101**	**139.07**	**159.1882**
RECOVERY %	**100.32389**	**100.216456**	**100.6172972**	**99.943773**	**99.25109**	**99.335379**	**99.492623**

Table 3.67 summary of accuracy results for amlodipine and losartan

Content%	**Amlodipine**	**Losartan**
40	99.94118018	100.32389
60	99.93709925	100.216456
80	100.339479	100.6172972
100	99.70867595	99.94377278
120	99.89048595	99.25108997
140	100.1602679	99.33537859
160	99.75692616	99.4926232
avg	**99.93589**	**99.3597**
STDEV	0.20546899	0.12258925
RSD	0.2056008	0.12337925

3. 2.5 Precision

i) Intraday Precision

Table 3.68 shows results of amlodipine and losartan mixed standard for intraday precision test.

Table 3.68 amlodipine and losartan mixed standard for intraday precision

No.	Amlodipine	Losartan
STD1	305882	4012771
SDT2	305538	4018013
STD3	306284	4020623
STD4	306195	4023237
STD5	306432	4023730
STD6	307485	4023393
Avg	306302.7	4020295
STDEV	531.99837	4289.006
RSD	0.0945927	**0.106684**

Tables numbered 3.69, 3.70 and 3.71 show intraday precission for 80%, 100% and 120% amlodipine, respectively, while tables numbered 3.72, 3.73 and 3.74 show intraday precission for 80%, 100% and 120% of losartan, respectively. Table 3.75 show the summary of the previous six tables, the average and RSD of each five assays of the three concentrations for each active ingredient.

Table 3.69 Intraday results for 80% amlodipine

	1st	2nd	3rd	4th	5th
1st trial	245546	245855	246197	245652	245724
2nd trial	245799	245976	245529	245771	245555
3rd trial	246051	245595	245593	245647	245453
AVG	245798.7	245808.7	245773	245690	245577.33
STDEV	252.5002	194.6801	368.5865	70.19259	136.87342
RSD	0.102726	0.0792	0.14997	0.02857	0.0557354
RECOVERY	**80.24699**	**80.25025**	**80.23861**	**80.21151**	**80.174729**
RECOVERY %	**100.3087**	**100.3128**	**100.2983**	**100.2644**	**100.21841**

Table 3.70 Intraday results for 100% amlodipine

	1st	2nd	3rd	4th	5th
1st trial	305594	305566	305429	305332	305346
2nd trial	306254	305252	305402	305285	305425
3rd trial	305822	305200	305283	305120	305249
AVG	305890	305339.3	305371.3	305245.7	305340
STDEV	335.2134	198.0135	77.68097	111.3388	88.153276
RSD	0.109586	0.06485	0.025438	0.036475	0.0288705
RECOVERY	**99.86517**	**99.68539**	**99.69583**	**99.65481**	**99.685605**
RECOVERY %	**99.86517**	**99.68539**	**99.69583**	**99.65481**	**99.685605**

Table 3.71 Intraday results for 120% amlodipine

	1st	2nd	3rd	4th	5th
1st trial	368185	367373	367089	367419	367707
2nd trial	367778	366658	366592	366662	366393
3rd trial	367579	368741	367469	366857	366441
AVG	367847.3	367590.7	367050	366979.3	366847
STDEV	308.8921	1058.422	439.7988	393.0475	745.16844
RSD	0.083973	0.287935	0.11982	0.107103	0.2031279
RECOVERY	**120.0928**	**120.009**	**119.8325**	**119.8094**	**119.76618**
RECOVERY %	**100.0773**	**100.0075**	**99.86038**	**99.84115**	**99.805149**

Table 3.72 Intraday results for 80% losartan

	1st	2nd	3rd	4th	5th
1st trial	3235264	3236189	3236730	3235188	3235630
2nd trial	3238516	3235695	3235766	3235175	3235280
3rd trial	3238175	3236565	3234174	3234661	3234838
AVG	3237318	3236150	3235557	3235008	3235249.3
STDEV	1787.256	436.3317	1290.794	300.5811	396.88957
RSD	0.055208	0.013483	0.039894	0.009292	0.0122677
RECOVERY	**80.52441**	**80.49534**	**80.48059**	**80.46694**	**80.472944**
RECOVERY %	**100.6555**	**100.6192**	**100.6007**	**100.5837**	**100.59118**

Table 3.73 Intraday results for 100% losartan

	1st	2nd	3rd	4th	5th
1st trial	4018877	4020599	4019421	4017536	4018824
2nd trial	4027233	4019952	4018614	4017988	4018037
3rd trial	4021455	4017152	4018422	4017555	4020447
AVG	4022522	4019234	4018819	4017693	4019102.7
STDEV	4278.904	1832.14	530.1123	255.6541	1228.9289
RSD	0.106374	0.045584	0.013191	0.006363	0.0305772
RECOVERY	**100.0554**	**99.97362**	**99.96329**	**99.93528**	**99.970342**
RECOVERY %	**100.0554**	**99.97362**	**99.96329**	**99.93528**	**99.970342**

Table 3.74 Intraday results for 120% losartan

	1st	2nd	3rd	4th	5th
1st trial	4789153	4784180	4785142	4785390	4784601
2nd trial	4786002	4786283	4783971	4780986	4784297
3rd trial	4785660	4785434	4785776	4784937	4782213
AVG	4786938	4785299	4784963	4783771	4783703.7
STDEV	1925.565	1057.98	915.7167	2422.493	1299.8728
RSD	0.040225	0.022109	0.019137	0.05064	0.0271729
RECOVERY	**119.0693**	**119.0286**	**119.0202**	**118.9906**	**118.98889**
RECOVERY %	**99.22445**	**99.19047**	**99.18351**	**99.1588**	**99.157406**

Table 3.75 Summery of intraday precision for amlodipine and losartan

	Amlodipine			Losartan		
	80 %	100 %	120 %	80 %	100 %	120 %
1st trial	100.3087	99.86517	100.0773	100.6555	100.0554	99.22445
2nd trial	100.3128	99.68539	100.0075	100.6192	99.97362	99.19047
3rd trial	100.2983	99.69583	99.86038	100.6007	99.96329	99.18351
4th trial	100.2644	99.65481	99.84115	100.5837	99.93528	99.1588
5th trial	100.2184	99.68561	99.80515	100.5912	99.97034	99.15741
Avg	**100.2805**	**99.71736**	**99.91829**	**100.6101**	**99.97958**	**99.18293**
STDEV	0.039597	0.084044	0.117626	0.028672	0.044988	0.027455
RSD	**0.039486**	**0.084282**	**0.117722**	**0.028498**	**0.044997**	**0.027682**

ii) Interday Precision

Table 3.76 shows results of amlodipine and losartan mixed standard for interday precision test.

Table 3.76 amlodipine and losartan mixed standard for interday precision

	Amlodipine			Losartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
STD1	305882	305324	305769	4012771	4029565	4029762
SDT2	305538	305802	305243	4018013	4028702	4029436
STD3	306284	305656	305906	4020623	4029254	4028864
STD4	306195	305568	305454	4023237	4028998	4027919
STD5	306432	305591	306036	4023730	4028993	4027343
STD6	307485	305431	306045	4023393	4028725	4028600
Avg	306302.667	305562	305742.2	4020294.5	4029040	4028654
STDEV	661.552014	167.9988	327.9679	4289.00631	328.2869	910.5108
RSD	**0.21597984**	**0.05498**	**0.107269**	**0.10668388**	**0.008148**	**0.022601**

Tables numbered 3.77, 3.78 and 3.79 show interday precission for 80%, 100% and 120% for both components, respectively.Table 3.80 shows the summary of interday precission, the average and RSD of each three assays of the three concentrations for each active ingredient.

Table 3.77 interday precission results for 80% of amlodipine and losartan

	Amlodipine			Losartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Trial 1	245546	245522	243579	3235264	3235472	3234131
Trial 2	245799	245642	243283	3238516	3235763	3233245
Trial 3	246051	245323	245495	3238175	3235489	3233713
Avg	245798.7	245495.7	244119	3237318	3235575	3233696
STDEV	252.5002	161.1221	1200.806	1787.256	163.3228	443.2351
RSD	0.102726	0.065631	0.491894	0.055208	0.005048	0.013707
Recovery	**80.24699**	**80.34234**	**79.84473**	**80.52441**	**80.30635**	**80.26741**

Table 3.78 interday precission results for 100% of amlodipine and losartan

	Amlodipine			Losartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Trial 1	305594	305243	305234	4018877	4018971	4017575
Trial 2	306254	305199	305402	4027233	4018252	4017564
Trial 3	305822	305280	304635	4021455	4017859	4016643
Avg	305890	305240.7	305090.3	4022522	4018361	4017261
STDEV	335.2134	40.55038	403.1778	4278.904	563.9081	534.9433
RSD	0.109586	0.013285	0.13215	0.106374	0.014033	0.013316
Recovery	**99.86517**	**99.65318**	**99.6041**	**100.0554**	**99.95189**	**99.92452**
Recovery%	**99.86517**	**99.65318**	**99.6041**	**100.0554**	**99.95189**	**99.92452**

Table 3.79 interday precission for 120% of amlodipine and losartan

	Amlodipine			Losartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Trial 1	368185	367369	366808	4789153	4785575	4782403
Trial 2	367778	366339	366275	4786002	4782701	4784376
Trial 3	367579	366298	366841	4785660	4781600	4783690
Avg	367847.3	366668.7	366641.3	**4786938**	**4783292**	**4783490**
STDEV	308.8921	606.8528	317.6828	1925.565	2052.344	1001.64
RSD	0.083973	0.165504	0.086647	0.040225	0.042907	0.02094
Recovery	**120.0928**	**119.9981**	**119.9185**	**119.0693**	**118.7204**	**118.7367**
Recovery%	**100.0773**	**99.99844**	**99.93206**	**99.22445**	**98.93367**	**98.94723**

Table 3.80 interday precission summery for both amlodipine and losartan

	Amlodipine			Losartan		
	80%	100%	120%	80%	100%	120%
Day 1	100.3087	99.86517	100.0773	100.0554	100.6555	99.22445
Day 2	100.4279	99.65318	99.99844	99.95189	100.3829	98.93367
Day 3	99.80591	99.6041	99.93206	99.92452	100.3343	98.94723
Avg	100.1809	99.70748	100.0026	99.97727	100.4576	99.03512
STDEV	0.330138	0.138748	0.072711	0.069024	0.173138	0.164111
RSD	0.329542	0.139155	0.072709	0.069039	0.172349	0.16571

3.2.6 Robustness:

The method was examined for robustness test under nine different conditions comparing the method output under each conditions with that of the optimized conditions and with permissible limits according to ICH, lastly the variation in method output was evaluated through calculation of RSD of the nine results obtained under the different nine conditions, the results shown in the followings.

i) Optimized conditions

Standard solution was injected six times while sample solution was injected three times under optimized conditions. Results of amlodipine and losartan standards are shown in Table 3.81 and Table 3.82, respectively, results of samples for both components are shown in Table 3.83

Table 3.81 Robustness results at optimum conditions for amlodipine Standard

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.36	305882	5677	1.65	6.061
SDT2	3.306	305538	5577	1.633	6.081
STD3	3.288	306284	5550	1.644	6.103
STD4	3.28	306195	5468	1.64	6.057
STD5	3.284	306432	5490	1.658	6.061
STD6	2.301	307485	5444	1.651	6.037
Avg	3.1365	306302.6667	5534.333333	1.646	6.066666667
STDEV	0.410376291	661.5520136	85.95968047	0.008876936	0.022642144
RSD	13.08389258	0.215979841	1.553207501	0.539303549	0.373222147

Table 3.82 Robustness results at optimum conditions for losartan Standards

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	4.46	4012771	9352	1.361	6.061
SDT2	4.402	4018013	9198	1.36	6.081
STD3	4.382	4020623	9255	1.361	6.103
STD4	4.375	4023237	9047	1.369	6.057
STD5	4.381	4023730	8997	1.371	6.061
STD6	4.392	4023393	9065	1.371	6.037
Avg	4.39866667	4020294.5	9152.3333	1.3655	6.066667
STDEV	4.38844444	4289.006307	138.08934	0.0053572	0.022642
RSD	1.00123783	**0.106683884**	1.5087884	0.3923279	0.373222

Table 3.83 Results of amlodipine and losartan sample at optimum conditions

	Amlodipine	Losartan
1st trial	305628	4017636
2nd trial	305314	4018188
3rd trial	305289	4018278
Avg.	305410.3333	4018034
STDEV	188.9188539	347.6032221
RSD	0.061857388	0.008651077
Recovery %	99.70867595	99.94377278

ii) 5^0C more

Standard solution was injected six times while sample solution was injected three times after the column temperature was raised up five degrees celsius, results of amlodipine and losartan standards are shown in Table 3.84 and Table 3.85, respectively; results of samples for both components are shown in Table 3.

Table 3.84 Results of amlodipine standard at increased temperature

No.	Ret.	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.341	304455	5545	1.645	5.827
SDT2	3.341	305629	5537	1.64	5.828
STD3	3.342	305747	5536	1.646	5.825
STD4	3.341	305769	5545	1.644	5.828
STD5	3.341	305422	5542	1.642	5.825
STD6	3.343	305633	5548	1.648	5.826
Avg	**3.3416**	**305442.5**	**5541.6**	**1.644**	**5.8264**
STDEV	0.000894427	499.1936498	5.128352562	0.003162278	0.001516575
RSD	0.026766435	0.163432937	0.092542814	0.192352656	0.026029368

Table 3.85 Results of losartan standard at increased temperature

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	4.414	4028844	8755	1.337	8.827
SDT2	4.413	4026889	8774	1.337	5.828
STD3	4.414	4026633	8767	1.338	5.825
STD4	4.413	4026336	8782	1.336	5.828
STD5	4.413	4025057	8774	1.335	5.825
STD6	4.415	4024558	8778	1.337	5.826
Avg	**4.4136**	**4026386.167**	**8775**	**1.3366**	**5.8264**
STDEV	0.00089443	**1514.33859**	5.5677644	0.0011402	0.001517
RSD	0.02026525	**0.037610366**	0.0634503	0.0853042	0.026029

Table 3.86 Results of amlodipine and losartan sample at increased temperature

	Amlodipine	Losartan
1st trial	305381	4018626
2nd trial	304888	4018395
3rd trial	305088	4017859
Avg.	305119	4018293.333
STDEV	247.9576577	393.4772319
RSD	0.081265886	0.009792148
Recovery %	99.89408809	99.79900504

iii) 5^{o}C less

Standard solution was injected six times while sample solution was injected three times after the column temperature was decreased five celsius degrees . Results of amlodipine and losartan standards are shown in Table 3.87 and Table 3.88, respectively; results of samples for both components are shown in Table 3.89.

Table 3.87 Results of amlodipine standard at decreased temperature

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.346	305731	5500	1.64	5.812
SDT2	3.346	306003	5502	1.642	5.812
STD3	3.346	305746	5509	1.641	5.815
STD4	3.346	304891	5508	1.64	5.813
STD5	3.344	305538	5534	1.648	5.826
STD6	3.344	305298	5540	1.644	5.826
Avg	**3.3452**	**305534.5**	**5518.6**	**1.643**	**5.8184**
STDEV	0.001095445	393.1598911	17.14059509	0.003162278	0.007021396
RSD	0.032746775	0.128679377	0.310596802	0.19246973	0.120675716

Table 3.88 Results of losartan standard at decreased temperature

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	4.417	4030375	8815	1.346	5.812
SDT2	4.417	4028931	8806	1.345	5.812
STD3	4.418	4028893	8803	1.343	5.815
STD4	4.417	4028777	8795	1.344	5.813
STD5	4.416	4029110	8789	1.342	5.826
STD6	4.416	4028332	8793	1.343	5.826
Avg	**4.4168**	**4029069.667**	**8797.2**	**1.3434**	**5.8184**
STDEV	0.00083666	**690.7629591**	7.0851958	0.0011402	0.007021
RSD	0.01894267	**0.017144478**	0.0805392	0.0848724	0.120676

Table 3.89 Results of amlodipine and losartan sample at decreased temperature

	Amlodipine	Losartan
1st trial	304401	4018633
2nd trial	304872	4019011
3rd trial	305334	4020027
Avg.	304869	4019223.667
STDEV	466.5072347	**720.9225562**
RSD	0.153018915	**0.017936861**
Recovery %	**99.78218499**	**99.75562597**

iv) 5% more flow

Standard solution was injected six times while sample solution was injected three times after increasing the flow rate 5% of its optimized value. Results of amlodipine and losartan standards are shown in Table 3.90 and Table 3.91, respectively; results of samples for both components are shown in table 3.92.

Table 3.90 Results of amlodipine standard at increased flow rate

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.409	291138	5527	1.656	5.584
SDT2	3.411	291982	5561	1.654	5.589
STD3	3.411	291620	5565	1.651	5.588
STD4	3.409	292435	5528	1.657	5.58
STD5	3.411	292386	5539	1.657	5.58
STD6	3.413	292599	5500	1.652	5.565
Avg	**3.411**	**292026.6667**	**5538.6**	**1.6542**	**5.5804**
STDEV	0.001414214	562.231862	26.46318197	0.002774887	0.009607289
RSD	0.04146038	0.192527576	0.477795507	0.167747998	0.172161295

Table 3.91 Results of losartan standard at increased flow rate

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	4.45	3820261	8830	1.335	5.584
SDT2	4.451	3820006	8845	1.335	5.589
STD3	4.451	3819021	8821	1.333	5.588
STD4	4.451	3819460	8798	1.334	5.58
STD5	4.452	3817877	8804	1.334	5.58
STD6	4.453	3818398	8799	1.33	5.565
Avg	**4.4516**	**3819170.5**	**8813.4**	**1.3332**	**5.5804**
STDEV	0.00089443	**923.3969352**	19.932386	0.0019235	0.009607
RSD	0.02009226	**0.024177945**	0.22616	0.1442798	0.172161

Table 3.92 Results of amlodipine and losartan sample at increased flow rate

	Amlodipine	Losartan
1st trial	290613	3803701
2nd trial	289886	3803160
3rd trial	289948	3803332
Avg.	290149	3803397.667
STDEV	403.0297756	**276.4133355**
RSD	0.138904417	**0.007267537**
Recovery %	**99.35702219**	**99.58700892**

v) 5% less flow

Standard solution was injected six times while sample solution was injected three times after decreasing the flow rate 5% of its optimized value. Results of amlodipine and losartan standards are shown in Table 3.93 and Table 3.94, respectively; results of samples for both components are shown in Table 3.95.

Table 3.93 Results of amlodipine standard at decreased flow rate

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.779	325090	5806	1.638	5.711
SDT2	3.781	325111	5793	1.643	5.716
STD3	3.778	323917	5808	1.635	5.733
STD4	3.779	325486	5817	1.643	5.736
STD5	3.778	323048	5831	1.647	5.747
STD6	3.781	325636	5791	1.649	5.73
Avg	**3.7794**	**324714.6667**	**5808**	**1.6434**	**5.7324**
STDEV	0.001516575	1015.461997	16.76305461	0.005366563	0.011193748
RSD	0.040127404	0.312724401	0.288620086	0.326552461	0.195271584

Table 3.94 Results of losartan standard at decreased flow rate

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	4.928	4267250	9338	1.326	5.711
SDT2	4.931	4258588	9355	1.325	5.716
STD3	4.931	4252943	9359	1.324	5.733
STD4	4.934	4250774	9322	1.327	5.736
STD5	4.934	4248987	9323	1.327	5.747
STD6	4.935	4243221	9340	1.328	5.73
Avg	**4.933**	**4253627.167**	**9339.8**	**1.3262**	**5.7324**
STDEV	0.00187083	**8350.084441**	17.311846	0.0016432	0.011194
RSD	0.03792477	**0.196305038**	0.1853556	0.1239004	0.195272

Table 3.95 Results of amlodipine and losartan sample at decreased flow rate

	Amlodipine	Losartan
1st trial	323774	4228419
2nd trial	320879	4227133
3rd trial	321155	4226116
Avg.	321936	4227222.667
STDEV	1597.725571	**1154.115389**
RSD	0.496286706	**0.027301978**
Recovery %	**99.14427436**	**99.37924743**

vi) 5% more organic solvent

Standard solution was injected six times while sample solution was injected three times after increasing of organic solvent in mobile phase 5% more than optimized value. Results of amlodipine and losartan standards are shown in Table 3.96 and Table 3.97, respectively; results of samples for both components are shown in Table 3.98.

Table 3.96 Results of amlodipine standard at increased organic solvent

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.339	305822	5542	1.654	5.903
SDT2	3.343	305332	5575	1.662	5.88
STD3	3.347	305243	5548	1.645	5.84
STD4	3.344	305568	5489	1.65	5.835
STD5	3.346	305438	5518	1.643	5.821
STD6	3.348	304635	5552	1.639	5.824
Avg	**3.3456**	**305339.6667**	**5536.4**	**1.6478**	**5.84**
STDEV	0.002073644	400.1023203	33.36615051	0.008871302	0.023674881
RSD	0.061981233	0.13103516	0.602668711	0.538372499	0.405391801

Table 3.97 Results of losartan standard at increased organic solvent

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	4.418	4021455	9003	1.365	5.903
SDT2	4.419	4017536	8928	1.354	5.88
STD3	4.421	4018971	8856	1.347	5.84
STD4	4.419	4028998	8868	1.349	5.835
STD5	4.418	4017564	8826	1.344	5.821
STD6	4.42	4016643	8810	1.341	5.824
Avg	**4.4194**	**4020194.5**	**8857.6**	**1.347**	**5.84**
STDEV	0.00114018	**4630.069751**	45.637704	0.0049497	0.023675
RSD	0.02579933	**0.115170292**	0.5152378	0.3674645	0.405392

Table 3.98 Results of amlodipine and losartan sample at increased organic solvent

	Amlodipine	Losartan
1st trial	305402	4018614
2nd trial	305249	4020447
3rd trial	305243	4018971
Avg.	305298	4019344
STDEV	90.11659115	**971.760773**
RSD	0.029517583	**0.024177099**
Recovery %	**99.98635399**	**99.97884431**

vii) 5% less organic solvent

Standard solution was injected six times while sample solution was injected three times after decreasing of organic solvent in mobile phase 5% more than optimized value. Results of amlodipine and losartan standards are shown in Table 3.99 and Table 3.100, respectively; results of samples for both components are shown in Table 3.101.

Table 3.99 Results of amlodipine standard at decreased organic solvent

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.6	248116	5660	1.653	5.64
SDT2	3.603	248275	5684	1.652	5.638
STD3	3.607	248376	5631	1.66	5.615
STD4	3.604	248825	5687	1.655	5.638
STD5	3.609	248397	5668	1.652	5.627
STD6	3.606	248206	5629	1.659	5.632
Avg	**3.6058**	**248365.8333**	**5659.8**	**1.6556**	**5.63**
STDEV	0.002387467	248.2429589	28.15492852	0.003781534	0.009565563
RSD	0.066211861	0.099950527	0.497454478	0.228408678	0.169903432

Table 3.100 Results of losartan standard at decreased organic solvent

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	4.698	3604305	9026	1.324	5.64
SDT2	4.701	3597856	8984	1.321	5.638
STD3	4.704	3606889	8977	1.32	5.615
STD4	4.702	3606213	8991	1.319	5.638
STD5	4.708	3606547	8975	1.323	5.627
STD6	4.705	3607003	9019	1.319	5.632
Avg	**4.704**	**3604802.167**	**8989.2**	**1.3204**	**5.63**
STDEV	0.00273861	**3542.028821**	17.810109	0.0016733	0.009566
RSD	0.05821881	**0.098258619**	0.1981279	0.1267283	0.169903

Table 3.101 Results of amlodipine and losartan sample at increased organic solvent

	Amlodipine	Losartan
1st trial	248027	3603696
2nd trial	247294	3595619
3rd trial	247466	3598812
Avg.	247595.6667	3599375.667
STDEV	383.3175359	**4067.89532**
RSD	0.154815931	**0.113016692**
Recovery %	**99.68990635**	**99.84946469**

viii) 3nm more

Standard solution was injected six times while sample solution was injected three times after increasing the 3nm more than the optimized detection wavelength. Results of amlodipine and losartan standards are shown in Table 3.102 and Table 3.103, respectively; results of samples for both components are shown in Table 3.104.

Table 3.102 Results of amlodipine standard at increased wavelength detection

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.353	247525	5447	1.635	5.735
SDT2	3.353	247562	5382	1.634	5.708
STD3	3.348	247202	5440	1.622	5.735
STD4	3.349	247248	5450	1.622	5.737
STD5	3.347	247273	5415	1.623	5.725
STD6	3.347	247441	5415	1.622	5.726
Avg	**3.3488**	**247375.1667**	**5420.4**	**1.6246**	**5.7262**
STDEV	0.00248998	153.8095142	26.4253666	0.005272571	0.011476062
RSD	0.074354393	0.062176619	0.48751691	0.324545767	0.200413224

Table 3.103 Results of losartan standard at increased wavelength detection

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	4.568	4483837	8981	1.322	5.713
SDT2	4.407	4498412	8767	1.324	5.739
STD3	4.379	4500731	8676	1.322	5.757
STD4	4.394	4499302	8683	1.325	5.754
STD5	4.408	4498573	8710	1.324	5.757
STD6	4.412	4496991	8668	1.322	5.739
Avg	**4.4**	**4496307.667**	**8700.8**	**1.3234**	**5.7492**
STDEV	0.01354622	**6230.18997**	40.233071	0.0013416	0.009391
RSD	0.30786857	**0.138562359**	0.4624066	0.1013783	0.163353

Table 3.104 Results of amlodipine and losartan sample at increased wavelength detection

	Amlodipine	Losartan
1st trial	245987	4482077
2nd trial	246449	4494799
3rd trial	246288	4482951
Avg.	246241.3333	4486609
STDEV	234.5087063	**7106.197577**
RSD	0.095235314	**0.158386826**
Recovery %	**99.54165434**	**99.78429709**

ix) 3nm less

Standard solution was injected six times while sample solution was injected three times after decreasing the 3nm more than the optimized detection wavelength. Results of amlodipine and losartan standards are shown in Table 3.105 and Table 3.106, respectively; results of samples for both components are shown in Table 3.107.

Table 3.105 Results of amlodipine standard at increased wavelength detection

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.486	406493	5602	1.644	5.713
SDT2	3.347	405969	5453	1.622	5.739
STD3	3.318	405973	5422	1.628	5.757
STD4	3.33	406144	5402	1.622	5.754
STD5	3.343	405973	5455	1.626	5.757
STD6	3.345	406143	5393	1.628	5.739
Avg	**3.3366**	**406115.8333**	**5425**	**1.6252**	**5.7492**
STDEV	0.012340989	203.051143	28.48683907	0.00303315	0.009391486
RSD	0.369867189	0.049998332	0.525103024	0.186632425	0.16335291

Table 3.106 Results of losartan standard at increased wavelength detection

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	4.418	3597000	8664	1.325	5.735
SDT2	4.417	3598263	8642	1.324	5.708
STD3	4.412	3598103	8659	1.321	5.735
STD4	4.413	3597771	8655	1.321	5.737
STD5	4.411	3597812	8630	1.323	5.725
STD6	4.411	3598449	8622	1.321	5.726
Avg	**4.4128**	**3597899.667**	**8641.6**	**1.322**	**5.7262**
STDEV	0.00248998	**511.6176948**	15.820872	0.0014142	0.011476
RSD	0.0564263	**0.014219899**	0.183078	0.1069753	0.200413

Table 3.107 Results of amlodipine and losartan sample at increased wavelength detection

	Amlodipine	Losartan
1st trial	403742	3586476
2nd trial	405222	3589349
3rd trial	404063	3588258
Avg.	404342.3333	3588027.667
STDEV	778.5373038	**1450.283535**
RSD	0.192544099	**0.040420077**
Recovery %	**99.56330193**	**99.7256177**

Summary of recovery for both components at the nine different conditions, average and RSD are shown in Table 3.108.

Table 3.108 amlodipine and losartan recovery at all robustness conditions

No	Condition	Amlodipine	Losartan
1	Optimized conditions	99.70867595	99.94377278
2	Mor 5 degree Celsius	99.89408809	99.79900504
3	less 5 degree Celsius	99.78218499	99.75562597
4	5% More flow rate	99.35702219	99.58700892
5	5% less flow rate	99.14427436	99.37924743
6	5% more Organic solvent	99.98635399	99.97884431
7	5% less Organic solvent	99.68990635	99.84946469
8	More 3 nm	99.54165434	99.7256177
9	Less 3 nm	99.56330193	99.78429709
	Avg	**99.65178656**	**99.75613845**
	STDEV	**0.262766244**	**0.182937003**
	RSD %	**0.263684428**	**0.183384207**

3.2.7 Assay:

Standard solution and sample solution were prepared as described in section (2-4-2-11); standard solution was injected six times results were shown in table 3.109, while sample solution was injected three times results were shown in table 3.110, the average of each was used for assay calculations.

Table 3.109 Assay mixed standard

	Amlodipine	Losartan
1	306148	4025019
2	306055	4024901
3	306105	4024350
4	306025	4024379
5	306018	4022922
6	306161	4023877
Avg	**306072.8**	**4024241.333**
STDEV	60.04331767	767.3415573
RSD	0.019617332	0.019067981

Table 3.110 Assay results of amlodipine and losartan

	Amlodipine	Losartan
1st trial	311928	4038939
2nd trial	311463	4038774
3rd trial	311577	4037043
AVG	311656	4038252
STDEV	242.3571744	1050.269965
RSD	0.077764322	0.026008034
Assay	**101.8241412**	**100.3481567**

3.3Amlodipine besylate and Atorvastatine calcium

3. 3.1 System Suitability

System suitability results for amlodipine besylate and atorvastatine calcium are shown in Table 3.111 and Table 3.112, respectively.

Table 3.111 System suitability results for amlodipine besylate

No.	Retention time	Area	Tailing factor	Resolution
1	2513267	0.516	13.166	1.406
2	2522982	0.514	13.139	1.412
3	2521604	0.518	13.106	1.408
4	2512317	0.517	13.137	1.406
5	2527706	0.514	13.114	1.409
6	2510037	0.511	13.122	1.412
Avg	**2518929**	**0.5148**	**13.1236**	**1.4094**
STDEV	**7473.36**	**0.00277**	**0.01433**	**0.00261**
RSD	**0.29669**	**0.53902**	**0.10918**	**0.18502**

Table 3.112 System suitability results for atorvastatine calcium

No.	Retention time	Area	Tailing factor	Resolution
1	2889085	4.066	13.166	1.226
2	2875557	4.055	13.139	1.225
3	2889141	4.063	13.106	1.233
4	2885150	4.065	13.137	1.23
5	2893686	4.068	13.114	1.233
6	2896797	4.064	13.122	1.233
Avg	**2888066**	**4.063**	**13.1236**	**1.2308**
STDEV	**8275.7**	**0.00485**	**0.01433**	**0.00349**
RSD	**0.28655**	**0.11931**	**0.10918**	**0.28379**

3. 3.2 Linearity, LOD and LOQ:

i) Amlodipine besylate

Table 3.113 shows linearity results for amlodipine besylate which then treated by XLSTAT-2015 program to predict linearity data that shown in Table 3.114, Table 3.115, Figure 3.15 and Figure 3.16.

Table 3.113 linearity result for amlodipine besylate

Conc µg/ml	40	60	80	100	120	140	160
1	965157	1474934	2008367	2527312	2963514	3541341	3951370
2	962120	1463757	2003623	2516640	2940860	3535883	3945785
3	962412	1471780	2010281	2516506	2954197	3522570	3968690
avg	**963229.666**	**1470157**	**2007423.7**	**2520153**	**2952857**	**3533265**	**3955281.7**
STDEV	**1675.49286**	**5762.5453**	**3427.7761**	**6200.526**	**11386.29**	**9655.5353**	**11943.0150**
RSD	**0.17394531**	**0.3919680**	**0.1707549**	**0.246037**	**0.38560**	**0.273275**	**0.301951**

Figure 3.15 shows the plot of average area versus concentrations for amlodipine besylate in µg/ml, the linear regression equation:

$$\text{Area} = -22484.21 + 125426.83 * \text{ng}$$

According to ICH guidelines, acceptance criteria is $R^2 \geq 0.997$.

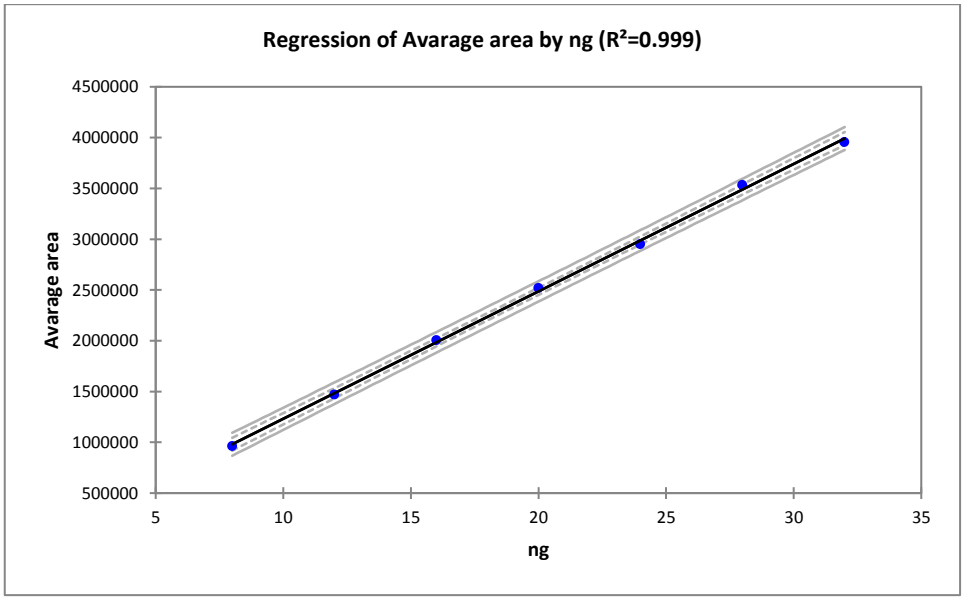

Figure 3.15 XL STAT 2015 plot of conc. in µg/ml vs average area of amlodipine besylate

Table 3.114 XL- STAT 2015 Goodness of fit statistics for amlodipine besylate

Goodness of fit statistics:	
Observations	7.000
Sum of weights	7.000
R^2	0.999
Adjusted R^2	0.999
MSE	1317850699.153
RMSE	36302.213

Table 3.115 XL STAT 2015 predicted area for amlodipine besylate

Observation	Weight	ng	Avarage area	Pred(area)
Obs1	1	8.000	963229.667	980930.405
Obs2	1	12.000	1470157.000	1482637.714
Obs3	1	16.000	2007423.667	1984345.024
Obs4	1	20.000	2520152.667	2486052.333
Obs5	1	24.000	2952857.000	2987759.643
Obs6	1	28.000	3533264.667	3489466.952
Obs7	1	32.000	3955281.667	3991174.262

Figure 3.16 is the a plot of average area versus predicted area for amlodipine , i.e. concentration Vs predicted concentration of amlodipine, acceptance limit for this graph is that slope ≥ 0.997

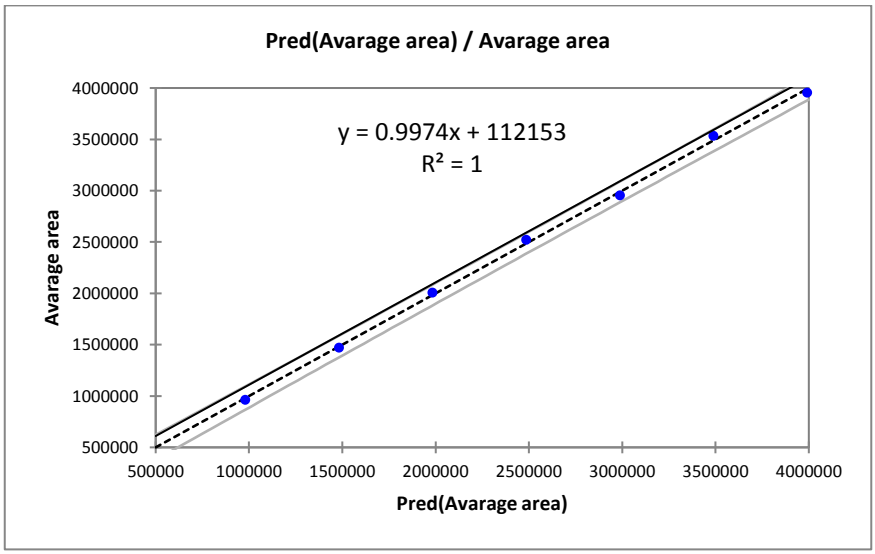

Figure 3.16 XL- STAT 2015 plot of area Vs Predicted area for amlodipine

Limit of Detection and Quantitation

LOD = 3.3* (SD/S).

LOD = 3.3* (36302/125426) = **0.96 ng = 0.05µg/ml**

LOD % (relative to target concentration) = 0.96*100/20 = 4.8%

LOQ = 10 * (SD/S)

= 10* (1552/91787) = **2.9 ng = 0.14µg/ml**

LOQ % (relative to target concentration) = 2.9 *100/20 = 14.5%

ii) Atorvastatine calcium

Table 3.116 shows linearity results for losartan potassium which then treated by XLSTAT-2015 program to predict linearity data that shown in Table 3.117, Table 3.118, Figure 3.17 and Figure 3.18.

Table 3.116 linearity result for atorvastatine calcium

Conc µg/ml	40	60	80	100	120	140	160
1	1134154	1707575	2305442	2852601	3367310	3972858	4599115
2	1130402	1704169	2305774	2836312	3362270	3991147	4546221
3	1142570	1703085	2310320	2824442	3380751	3978620	4541278
avg	**1135708.667**	**1704943**	**2307178.667**	**2837785**	**3370110.333**	**3980875**	**4562204.667**
STDEV	**6231.195498**	**1209541.08**	**2725.534321**	**14137.17146**	**9553.441282**	**9350.703129**	**32060.68999**
RSD	**0.548661438**	**70.94319753**	**0.118132781**	**0.49817627**	**0.283475624**	**0.234890649**	**0.702745544**

Figure 3.17 shows the plot of average area versus concentrations for atorvastatine calcium in µg/ml, the linear regression equation:

$$\text{Area} = 4421.54 + 141913.25 * \text{ng}$$

According to ICH guidelines, acceptance criteria is $R^2 \geq 0.997$

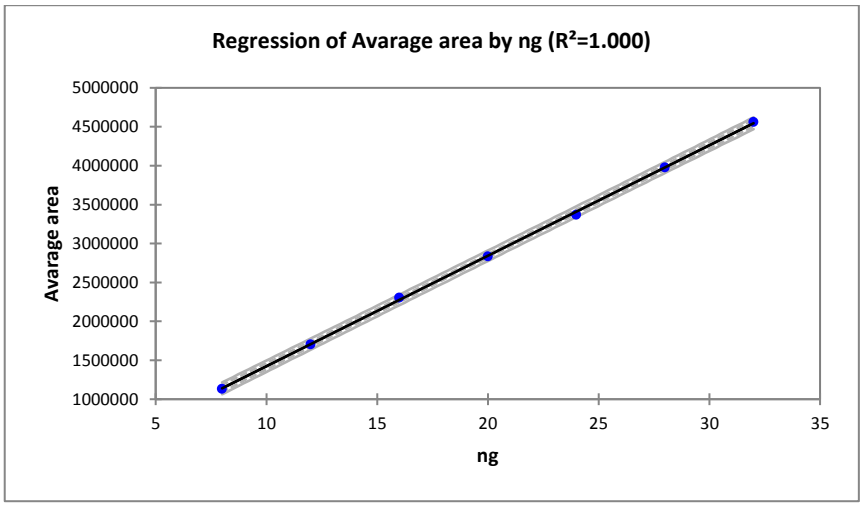

Figure 3.17 XL- STAT 2015 plot of conc. in µg/ml Vs average area of atorvastatine calcium

Table 3.117 XL- STAT 2015 Goodness of fit statistics of atorvastatine calcium

Goodness of fit statistics:	
Observations	7.000
Sum of weights	7.000
R^2	1.000
Adjusted R^2	1.000
MSE	596065905.505
RMSE	24414.461

Table 3.118 XL- STAT 2015 predicted area for atorvastatine calcium

Observation	Weight	ng	Average area	Predicted area
Obs1	1	8.000	1135708.667	1139727.512
Obs2	1	12.000	1704943.000	1707380.500
Obs3	1	16.000	2307178.667	2275033.488
Obs4	1	20.000	2837785.000	2842686.476
Obs5	1	24.000	3370110.333	3410339.464
Obs6	1	28.000	3980875.000	3977992.452
Obs7	1	32.000	4562204.667	4545645.440

Figure 3.18 is the plot of average area versus predicted area for atorvastatine calcium , i.e. concentration Vs predicted concentration of atorvastatine calcium , acceptance limit for this graph is that slope ≥ 0.997.

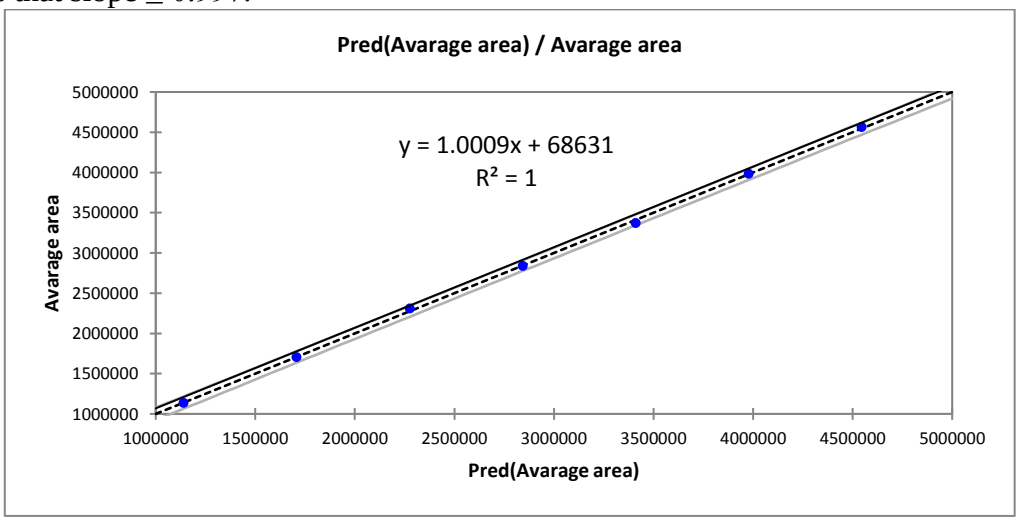

Figure 3.18 XL- STAT 2015 Graph of (area) Vs (Predicted area) for atorvastatine calcium

Limit of Detection and Quantitation

LOD = 3.3* (SD/S).

LOD = 3.3* (24414/141913) = **0.57 ng = 0.03µg/ml**

LOD % (relative to target concentration) = 0.57*100/20 = 0.8%

LOQ = 10 * (SD/S).

LOQ = 10* (24414/141913) = **1.7 ng = 0.09µg/ml**

LOQ % (relative to target concentration) = 1.7*100/20 = 8.5%

3.3.3 Specificity

Figure 3.19 Figure 3.20 and figure 3.21 shows the specificity chromatograms for placebo, sample and standard, respectively; for amlodipine besylate and atorvastatine calcium .

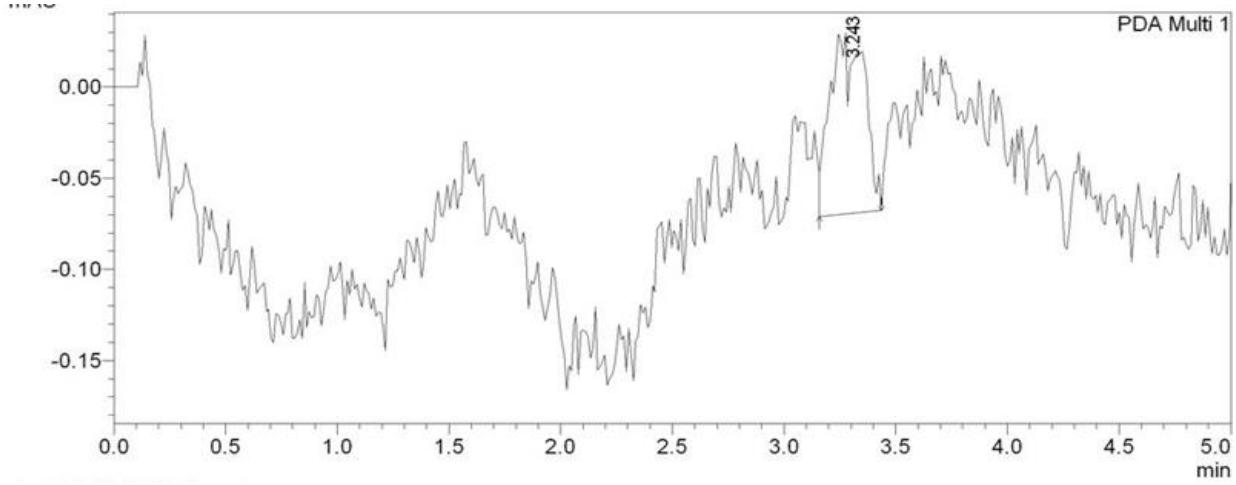

Figure 3.19 chromatogram for the Placebo of hydrochlorothiazide and atorvastatine calcium

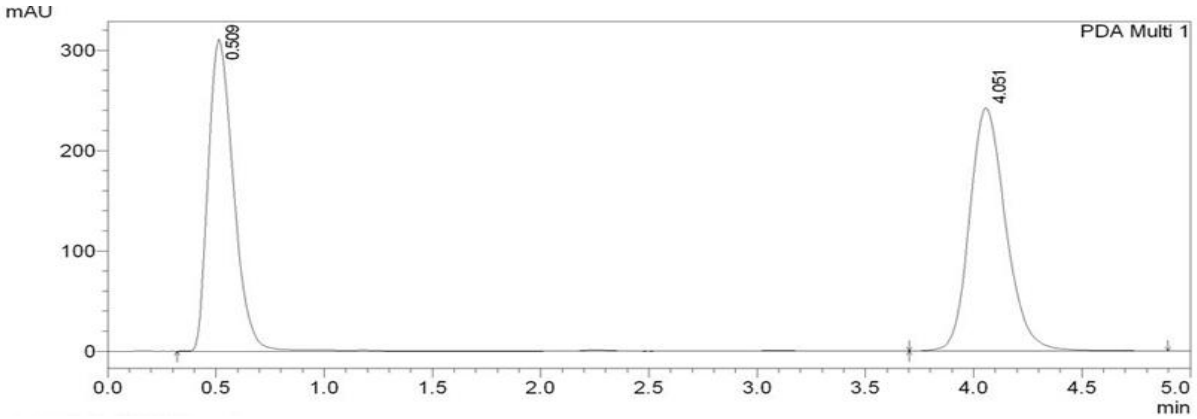

figure 3.20 chromatogram for the sample of amlodipine besylate and atorvastatine calcium

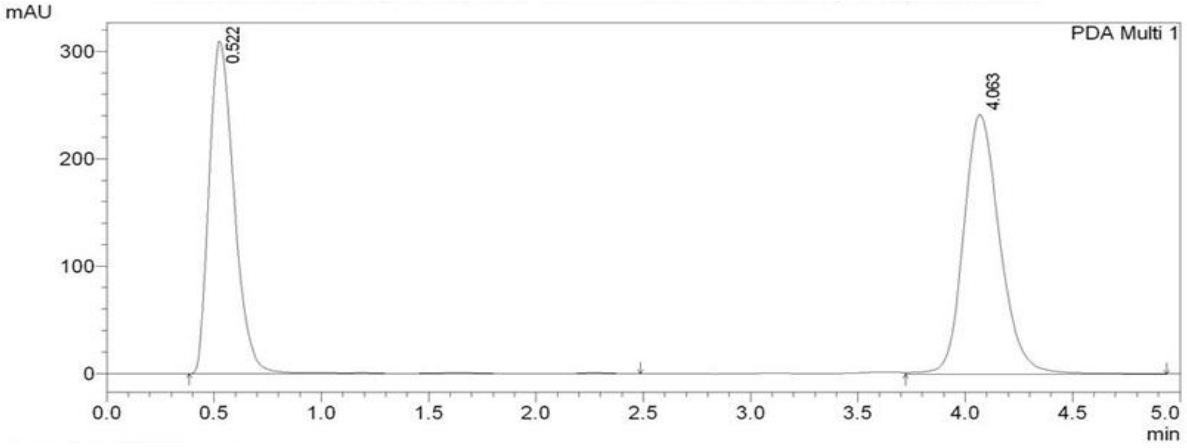

figure 3.21 chromatogram for mixed standard of amlodipine besylate and atorvastatine calcium

3.3.4 Accuracy

Table 3.119 shows the results of mixed standard of amlodipine besylate and atorvastatine calcium, while the accuracy results for samples are shown in Table 3.120 and Table 3.121, respectively; summary of accuracy results for both components is shown in Table 3.122.

Table 3.119 Results of amlodipine and atorvastatine standard for accuracy test

No.	Amlodipine	atorvastatine
STD1	2513267	2889085
SDT2	2522982	2875557
STD3	2521604	2889141
STD4	2512317	2885150
STD5	2527706	2893686
STD6	2510037	2896797
Avg	2518929	2888066
STDEV	7473.36	8275.7
RSD	0.29669	0.28655

Table 3.120 Accuracy results for amlodipine

Content	40	60	80	100	120	140	160
1	995515	1500310	2017083	2514448	3004945	3517389	4017072
2	997459	1494550	2005871	2527312	3032338	3539855	4035730
3	1001646	1517500	2013495	2521604	3007200	3540901	4053361
avg	998206.7	1504120	2012150	2521121	3014828	3532715	4035388
STDEV	3133.137	11939.96	5725.791	6445.568	15206.25	13283.01	18146.92
RSD	0.313877	0.793817	0.284561	0.255663	0.504382	0.376	0.449695
RECOVERY	39.62821	59.71267	79.88115	100.087	119.6869	140.2467	160.2025
RECOVERY %	99.07054	99.52112	99.85144	100.087	99.73906	100.1762	100.1266

Table 3.121 Accuracy results for atorvastatine

Content	40	60	80	100	120	140	160
1	1138153	1707575	2294067	2870220	3468949	4002251	4637322
2	1130028	1683192	2281049	2852601	3468132	4011091	4660134
3	1134154	1704169	2296992	2889141	3418864	4002251	4633292
avg	1134112	1698312	2290703	2870654	3451982	4005198	4643583
STDEV	4062.665	13204.58	8487.277	18273.87	28683.65	5103.776	14474.81
RSD	0.358224	0.777512	0.37051	0.636575	0.830933	0.127429	0.311716
RECOVERY	39.26889	58.80447	79.31614	99.3971	119.5257	138.681	160.7852
RECOVERY %	98.17224	98.00745	99.14518	99.3971	99.60476	99.05782	100.4907

Table 3.122 summary of accuracy results for amlodipine and atorvastatine

Content%	Amlodipine	Losartan
40	99.07054	98.17224
60	99.52112	98.00745
80	99.85144	99.14518
100	100.087	99.3971
120	99.73906	99.60476
140	100.1762	99.05782
160	100.1266	100.4907
avg	100.0139	99.71777
STDEV	0.239347	0.723114
RSD	0.239314	0.725161

3.3.5 Precision

i) Intraday Precision

Table 3.123 shows results of amlodipine and atorvastatine mixed standard for intraday precision test.

Table 3.123 amlodipine and atorvastatine mixed standard for intraday precision

No.	Amlodipine	Losartan
STD1	2506863	2607554
SDT2	2500209	2632440
STD3	2491301	2635480
STD4	2503952	2654284
STD5	2503451	2594729
STD6	2490447	2599983
Avg	**2499371**	**2620745**
STDEV	6917.036	23497.46
RSD	**0.276751**	**0.896595**

Tables numbered 3.124, 3.125 and 3.126 show intraday precision for 80%, 100% and 120% amlodipine, respectively; while tables numbered 3.127, 3.128 and 3.129 shows intraday precision for 80%, 100% and 120% of atorvastatine, respectively. Table 3.130 shows the summary of the previous six tables, the average and RSD of each five assays of the three concentrations for each active ingredient.

Table 3.124 Intraday results for 80% amlodipine

	1st	2nd	3rd	4th	5th
1st trial	2015551	2016382	2029175	2043419	2023782
2nd trial	2014852	2017589	1991047	2028472	2032094
3rd trial	2005164	2022245	1996649	2028034	2025049
AVG	2011856	2018739	2005624	2033308	2026975
STDEV	5805.683	3095.964	20587.49	8758.832	4478.219
RSD	0.288574	0.153361	1.026488	0.430768	0.220931
RECOVERY	**80.4945**	**80.76988**	**80.24515**	**81.35282**	**81.09942**
RECOVERY %	**100.6181**	**100.9624**	**100.3064**	**101.691**	**101.3743**

Table 3.125 Intraday results for 100% amlodipine

	1st	2nd	3rd	4th	5th
1st trial	2518439	2506680	2500610	2490160	2517999
2nd trial	2518225	2496668	2506680	2514266	2516796
3rd trial	2488994	2494821	2508722	2515974	2521125
AVG	2508553	2499390	2505337	2506800	2518640
STDEV	16938.64	6380.797	4219.384	14435.95	2234.552
RSD	0.675236	0.255294	0.168416	0.575871	0.088721
RECOVERY	**100.3674**	**100.0008**	**100.2387**	**100.2973**	**100.771**
RECOVERY %	**100.3674**	**100.0008**	**100.2387**	**100.2973**	**100.771**

Table 3.126 Intraday results for 120% amlodipine

	1st	2nd	3rd	4th	5th
1st trial	3002547	3004452	3004703	2998621	3011428
2nd trial	2998621	3023990	3000602	3002547	2990476
3rd trial	2985912	3001587	3006971	3032910	3004452
AVG	2995693	3010010	3004092	3011359	3002119
STDEV	8695.357	12191.77	3228.162	18766.37	10669.11
RSD	0.290262	0.405041	0.107459	0.623186	0.355386
RECOVERY	**119.8579**	**120.4307**	**120.1939**	**120.4847**	**120.115**
RECOVERY %	**99.88159**	**100.3589**	**100.1616**	**100.4039**	**100.0958**

Table 3.127 Intraday results for 80% atorvastatine

	1st	2nd	3rd	4th	5th
1st trial	2103382	2082866	2101959	2111948	2105111
2nd trial	2084940	2093160	2098006	2080581	2093998
3rd trial	2091580	2080871	2094325	2119427	2125542
AVG	2093301	2085632	2098097	2103985	2108217
STDEV	9340.63	6595.023	3817.808	20610.82	15999.73
RSD	0.446215	0.316212	0.181965	0.979609	0.758922
RECOVERY	**79.87426**	**79.58166**	**80.05726**	**80.28196**	**80.44342**
RECOVERY %	**99.84282**	**99.47707**	**100.0716**	**100.3524**	**100.5543**

Table 3.128 Intraday results for 100% atorvastatine

	1st	2nd	3rd	4th	5th
1st trial	2622332	2620658	2618492	2610852	2606971
2nd trial	2638408	2608610	2620658	2613081	2602349
3rd trial	2598313	2620947	2600188	2637364	2617416
AVG	2619684	2616738	2613113	2620432	2608912
STDEV	20178.2	7040.826	11245.36	14705.55	7718.759
RSD	0.770253	0.269069	0.430344	0.561188	0.295861
RECOVERY	**99.95953**	**99.84712**	**99.70877**	**99.98807**	**99.54849**
RECOVERY %	**99.95953**	**99.84712**	**99.70877**	**99.98807**	**99.54849**

Table 3.129 Intraday results for 120% atorvastatine

	1st	2nd	3rd	4th	5th
1st trial	3002547	3004452	3004703	2998621	3011428
2nd trial	2998621	3023990	3000602	3002547	2990476
3rd trial	2985912	3001587	3006971	3032910	3004452
AVG	2995693	3010010	3004092	3011359	3002119
STDEV	8695.357	12191.77	3228.162	18766.37	10669.11
RSD	0.290262	0.405041	0.107459	0.623186	0.355386
RECOVERY	**119.8579**	**120.4307**	**120.1939**	**120.4847**	**120.115**
RECOVERY %	**99.88159**	**100.3589**	**100.1616**	**100.4039**	**100.0958**

Table 3.130 Summery of intraday precision for amlodipine and atorvastatine

	Hydrochlorothiazide			Valsartan		
	80 %	100 %	120 %	80 %	100 %	120 %
1st trial	100.6181	100.3674	99.88159	99.84282	100.3674	99.88159
2nd trial	100.9624	100.0008	100.3589	99.47707	100.0008	100.3589
3rd trial	100.3064	100.2387	100.1616	100.0716	100.2387	100.1616
4th trial	101.691	100.2973	100.4039	100.3524	100.2973	100.4039
5th trial	101.3743	100.771	100.0958	100.5543	100.771	100.0958
Avg	**100.9904**	**100.335**	**100.1804**	**100.0596**	**100.335**	**100.1804**
STDEV	0.55799	0.279967	0.211321	0.423361	0.279967	0.211321
RSD	**0.552518**	**0.279032**	**0.21094**	**0.423109**	**0.279032**	**0.21094**

ii) Interday Precision

Table 3.131 show results of amlodipine and atorvastatine mixed standard for interday precision test.

Table 3.131 amlodipine and atorvastatine mixed standard for interday precision

	Hydrochlorothiazide			Valsartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
STD1	2506863	2519516	2506953	2607554	2482850	2500740
SDT2	2500209	2526248	2500422	2632440	2482846	2486406
STD3	2491301	2509770	2498521	2635480	2485784	2513853
STD4	2503952	2505280	2502529	2654284	2504972	2509086
STD5	2503451	2492881	2537015	2594729	2494504	2491759
STD6	2490447	2505377	2492846	2599983	2490014	2480246
Avg.	**2499370.5**	**2509845**	**2506381**	**2620745**	**2490162**	2497015
STDEV	6917.03612	11757.311	15710.46	23497.4601	8536.592	13148.9824
RSD	0.27675113	0.4684476	0.626818	0.89659467	0.342813	0.52658804

Tables numbered 3.132, 3.133 and 3.134 shows intraday precision for 80%, 100% and 120% for both components, respectively.Table 3.135 shows the summary of interday precision, the average and RSD of each three assays of the three concentrations for each active ingredient.

Table 3.132 interday precision results for 80% of amlodipine and atorvastatine

	Hydrochlorothiazide			Valsartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
STD1	2015551	2014179	2021528	2103382	2005142	2005660
SDT2	2014852	2012439	2018919	2084940	1999989	1990947
STD3	2005164	2009360	2028232	2091580	2002494	2026246
STD4	2011856	2011993	2022893	2093301	2002542	2007618
STD5	5805.683	2440.31	4804.21	9340.63	2576.83	17730.7
STD6	0.288574	0.12129	0.23749	0.446215	0.12868	0.88317
Avg.	**80.4945**	**80.164**	**80.71**	**79.87426**	**80.418**	**80.401**
STDEV	**100.6181**	**100.205**	**100.89**	**99.84282**	**100.52**	**100.5**
RSD	2015551	2014179	2021528	2103382	2005142	2005660

Table 3.133 interday precision results for 100% of amlodipine and atorvastatine

	Hydrochlorothiazide			Valsartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
STD1	3002547	3032710	3015390	3153711	2979775	3016772
SDT2	2998621	3009626	3044265	3154297	2998296	3009591
STD3	2985912	2996682	3023929	3147393	2980304	3001592
STD4	**2995693**	**3E+06**	**3E+06**	3151800	2986125	3009318
STD5	8695.357	18250.3	14833.7	3828.092	10543.7	7593.67
STD6	0.290262	0.60572	0.48991	0.121457	0.35309	0.25234
Avg.	**119.8579**	**120.5506**	**121.145**	**120.2635**	**119.92**	**120.52**
STDEV	**99.88159**	**100.4588**	**100.9541**	**100.2196**	**99.93076**	**100.4305**
RSD	3002547	3032710	3015390	3153711	2979775	3016772

Table 3.134 interday precision for 120% of amlodipine and atorvastatine

	Hydrochlorothiazide			Valsartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
STD1	3002547	3032710	3015390	3153711	2979775	3016772
SDT2	2998621	3009626	3044265	3154297	2998296	3009591
STD3	2985912	2996682	3023929	3147393	2980304	3001592
STD4	**2995693**	**3E+06**	**3E+06**	3151800	2986125	3009318
STD5	8695.357	18250.3	14833.7	3828.092	10543.7	7593.67
STD6	0.290262	0.60572	0.48991	0.121457	0.35309	0.25234
Avg.	**119.8579**	**120.5506**	**121.145**	**120.2635**	**119.92**	**120.52**
STDEV	**99.88159**	**100.4588**	**100.9541**	**100.2196**	**99.93076**	**100.4305**
RSD	3002547	3032710	3015390	3153711	2979775	3016772

Table 3.135 interday precision summery for both amlodipine and atorvastatine

	Amlodipine			**atorvastatine**		
	80%	100%	120%	80%	100%	120%
Day 1	100.62	100.37	99.88	99.84	99.96	100.22
Day 2	100.21	99.65	100.46	100.52	100.10	99.93
Day 3	100.89	100.15	100.95	100.50	99.836	100.43
Avg	**100.57**	**100.06**	100.71	**100.29**	**99.965**	100.19
STDEV	0.344	0.366	0.35	0.38637	0.1318	0.2508
RSD	**0.342**	**0.366**	0.35	**0.38526**	**0.131851**	0.2504

3.3.6 Robustness:

The method was examined for robustness test under nine different conditions comparing the method output under each conditions with that of the optimized conditions and with permissible limits according to ICH, lastly the variation in method output was evaluated through calculation of RSD of the nine results obtained under the different nine conditions, the results shown in the followings.

i) Optimized conditions

Standard solution was injected six times while sample solution was injected three times under optimized conditions. Results of amlodipine and atorvastatine standards are shown in Table 3.136 and Table 3.137, respectively; results of samples for both components are shown in Table 3.138.

Table 3.136 Robustness results at optimum conditions for amlodipine Standard

No.	Ret. Time	Area	Tailing factor	Resolution
STD1	0.522	2516058	1.357	13.276
SDT2	0.519	2515870	1.36	13.113
STD3	0.52	2502738	1.363	13.139
STD4	0.512	2493963	1.367	13.197
STD5	0.526	2491972	1.371	13.193
STD6	0.51	2505280	1.417	13.206
Avg	0.518167	**2504314**	1.3725	13.18733
STDEV	0.00608	10337.77	0.022358	0.056916
RSD	1.173372	0.412799	1.62903	0.431598

Table 3.137 Robustness results at optimum conditions for atorvastatine Standard

No.	Ret. Time	Area	Tailing factor	Resolution
STD1	4.185	2535899	1.186	13.276
SDT2	4.09	2511934	1.176	13.113
STD3	4.103	2531898	1.184	13.139
STD4	4.101	2499099	1.188	13.197
STD5	4.115	2494782	1.182	13.193
STD6	4.064	2504972	1.216	13.206
Avg	4.109667	**2513097**	1.188667	13.18733
STDEV	0.040732	17159.02	0.01401	0.056916
RSD	0.991118	0.682784	1.178591	0.431598

Table 3.138 Results of amlodipine and atorvastatine sample at optimum conditions

	Amlodipine	atorvastatine
1st trial	2502269	2507937
2nd trial	2500876	2498045
3rd trial	2502269	2507937
Avg.	**2501805**	**2504640**
STDEV	804.2489	5711.149
RSD	0.032147	0.228023
Recovery %	**99.89982**	**99.66346**

ii) 5ºC more

Standard solution was injected six times while sample solution was injected three times after the column temperature was raised up five degrees celsius, Results of amlodipine and atorvastatine standards are shown in Table 3.139 and Table 3.140, respectively; results of samples for both components are shown in Table 3.141.

Table 3.139 Results of amlodipine standard at increased temperature

No.	Ret. Time	Area	Tailing factor	Resolution
STD1	0.52	2498858	1.368	13.207
SDT2	0.515	2480690	1.374	13.222
STD3	0.521	2498170	1.37	13.214
STD4	0.514	2491999	1.375	13.238
STD5	0.528	2495361	1.372	13.218
STD6	0.522	2497877	1.371	13.259
Avg	**0.52**	**2493826**	**1.3724**	**13.2302**
STDEV	0.005701	6911.593	0.002074	0.018499
RSD	1.096323	0.277148	0.151096	0.139821

Table 3.140 Results of atorvastatine standard at increased temperature

No.	Ret. Time	Area	Tailing factor	Resolution
STD1	4.122	2525736	1.19	13.207
SDT2	4.117	2506393	1.195	13.222
STD3	4.12	2523318	1.196	13.214
STD4	4.114	2505410	1.191	13.238
STD5	4.127	2502597	1.188	13.218
STD6	4.123	2498013	1.194	13.259
Avg	**4.1202**	**2510245**	**1.1928**	**13.2302**
STDEV	0.00507	11464.76	0.003271	0.018499
RSD	0.123041	0.456719	0.274236	0.139821

Table 3.141 Results of amlodipine and atorvastatine sample at increased temperature

	Amlodipine	atorvastatine
1st trial	2494731	2507188
2nd trial	2506953	2500740
3rd trial	2493473	2491389
Avg.	2498386	2499772
STDEV	7446.143	7943.827
RSD	0.298038	0.317782
Recovery %	**100.1828**	**99.58282**

iii) 5°C less

Standard solution was injected six times while sample solution was injected three times after the column temperature was decreased five celsius degrees . Results of amlodipine and atorvastatine standards are shown in Table 3.142 and Table 3.143, respectively; results of samples for both components are shown in Table 3.144.

Table 3.142 Results of amlodipine standard at decreased temperature

No.	Ret. Time	Area	Tailing factor	Resolution
STD1	0.528	2492531	1.369	13.209
SDT2	0.528	2497687	1.371	13.188
STD3	0.531	2486844	1.369	13.201
STD4	0.513	2490766	1.376	13.227
STD5	0.522	2494101	1.371	13.22
STD6	0.531	2492757	1.369	13.196
Avg	**0.525**	**2492448**	**1.3712**	**13.2064**
STDEV	0.007649	3592.098	0.002864	0.016471
RSD	1.456863	0.144119	0.208836	0.124721

Table 3.143 Results of atorvastatine standard at decreased temperature

No.	Ret. Time	Area	Tailing factor	Resolution
STD1	4.127	2509811	1.196	13.209
SDT2	4.125	2501949	1.187	13.188
STD3	4.128	2493785	1.191	13.201
STD4	4.11	2509281	1.194	13.227
STD5	4.12	2511261	1.188	13.22
STD6	4.133	2497274	1.191	13.196
Avg	**4.1232**	**2503894**	**1.1902**	**13.2064**
STDEV	0.008758	7322.604	0.002775	0.016471
RSD	0.212404	0.292449	0.233145	0.124721

Table 3.144 Results of amlodipine and atorvastatine sample at decreased temperature

	Hydrochlorothiazide	Valsartan
1st trial	2498099	2505265
2nd trial	2499544	2500290
3rd trial	2480503	2502526
Avg.	**2492715**	**2502694**
STDEV	**10600.84**	**2491.734**
RSD	**0.425273**	**0.099562**
Recovery %	**100.0107**	**99.95208**

iv) 5% more flow

Standard solution was injected six times while sample solution was injected three times after increasing the flow rate 5% of its optimized value. Results of amlodipine and atorvastatine standards are shown in Table 3.145 and Table 3.146, respectively; results of samples for both components are shown in Table 3.147.

Table 3.145 Results of amlodipine standard at increased flow rate

No.	Ret. Time	Area	Tailing factor	Resolution
STD1	0.499	2416926	1.376	13.162
SDT2	0.505	2423645	1.371	13.31
STD3	0.51	2425839	1.378	13.301
STD4	0.516	2447672	1.375	13.332
STD5	0.505	2428512	1.367	13.277
STD6	0.512	2435497	1.377	13.229
Avg	**0.5096**	**2429682**	**1.3736**	**13.2898**
STDEV	0.004722	10702.83	0.004561	0.03929
RSD	0.926666	0.440503	0.332025	0.29564

Table 3.146 Results of atorvastatine standard at increased flow rate

No.	Ret. Time	Area	Tailing factor	Resolution
STD1	3.999	2475226	1.19	13.162
SDT2	4.012	2480449	1.19	13.31
STD3	4.013	2492851	1.178	13.301
STD4	4.015	2480049	1.19	13.332
STD5	4.004	2481173	1.186	13.277
STD6	4.015	2492052	1.192	13.229
Avg	**4.0118**	**2483633**	**1.1872**	**13.2898**
STDEV	0.00455	7149.311	0.005586	0.03929
RSD	0.113409	0.287857	0.470493	0.29564

Table 3.147 Results of amlodipine and atorvastatine sample at increased flow rate

	Hydrochlorothiazide	Valsartan
1st trial	2423306	2487806
2nd trial	2438904	2479933
3rd trial	2420451	2477429
Avg.	2427554	2481723
STDEV	9932.789	5415.045
RSD	0.409169	0.218197
Recovery %	**99.91241**	**99.92307**

v) 5% less flow

Standard solution was injected six times while sample solution was injected three times after decreasing the flow rate 5% of its optimized value. Results of amlodipine and atorvastatine standards are shown in Table 3.148 and Table 3.149, respectively; results of samples for both components are shown in Table 3.150.

Table 3.148 Results of amlodipine standard at decreased flow rate

No.	Ret. Time	Area	Tailing factor	Resolution
STD1	0.538	2579962	1.376	13.33
SDT2	0.532	2587709	1.367	13.315
STD3	0.545	2579692	1.369	13.332
STD4	0.54	2587884	1.369	13.331
STD5	0.544	2582061	1.362	13.503
STD6	0.548	2584731	1.37	13.346
Avg	**0.5418**	**2583673**	**1.3674**	**13.3654**
STDEV	0.006181	3670.412	0.003209	0.0777
RSD	1.140756	0.142062	0.234705	0.581352

Table 3.149 Results of atorvastatine standard at decreased flow rate

No.	Ret. Time	Area	Tailing factor	Resolution
STD1	4.26	2656749	1.638	13.33
SDT2	4.255	2690044	1.184	13.315
STD3	4.268	2670148	1.189	13.332
STD4	4.263	2683147	1.185	13.331
STD5	4.269	2673474	1.18	13.503
STD6	4.277	2689916	1.186	13.346
Avg	**4.2664**	**2677246**	**1.1848**	**13.3654**
STDEV	0.008112	12991.35	0.003271	0.0777
RSD	0.19013	0.485251	0.276088	0.581352

Table 3.150 Results of amlodipine and atorvastatine sample at decreased flow rate

	Hydrochlorothiazide	Valsartan
1st trial	2588703	2676342
2nd trial	2578856	2680690
3rd trial	2570607	2669400
Avg.	2579389	2675477
STDEV	9059.752	5694.45
RSD	0.351236	0.212839
Recovery %	**99.83417**	**99.93392**

vi) 5% more organic solvent

Standard solution was injected six times while sample solution was injected three times after increasing of organic solvent in mobile phase 5% more than optimized value. Results of amlodipine and atorvastatine standards are shown in Table 3.151 and Table 3.152, respectively; results of samples for both components are shown in table 3.153.

Table 3.151 Results of amlodipine standard at increased organic solvent

No.	Ret. Time	Area	Tailing factor	Resolution
STD1	0.533	2499627	1.371	5.903
SDT2	0.524	2508517	1.372	5.88
STD3	0.522	2494101	1.371	5.84
STD4	0.521	2492975	1.391	5.835
STD5	0.523	2493263	1.399	5.821
STD6	0.528	2500771	1.391	5.824
Avg	**0.5236**	**2498209**	**1.3848**	**5.84**
STDEV	0.002702	6059.089	0.012578	0.023675
RSD	0.516014	0.242537	0.908273	0.405392

Table 3.152 Results of atorvastatine standard at increased organic solvent

No.	Ret. Time	Area	Tailing factor	Resolution
STD1	4.134	2525955	1.184	13.361
SDT2	4.071	2539790	1.18	13.354
STD3	4.12	2511261	1.188	13.22
STD4	4.125	2497245	1.189	13.443
STD5	4.139	2500465	1.189	13.488
STD6	3.989	2494842	1.188	13.276
Avg	**4.0888**	**2511593**	**1.1868**	**13.3562**
STDEV	0.061402	17954.06	0.003834	0.111683
RSD	1.501711	0.714848	0.323058	0.836192

Table 3.153 Results of amlodipine and atorvastatine sample at increased organic solvent

	Amlodipine	atorvastatine
1st trial	2504732	2503263
2nd trial	2506127	2490843
3rd trial	2512956	2495148
Avg.	2507938	2496418
STDEV	4401.05	6306.645
RSD	0.175485	0.252628
Recovery %	**100.3895**	**99.3958**

vii) 5% less organic solvent

Standard solution was injected six times while sample solution was injected three times after decreasing of organic solvent in mobile phase 5% more than optimized value. Results of amlodipine and atorvastatine standards are shown in Table 3.154 and Table 3.155, respectively; results of samples for both components are shown in Table 3.156.

Table 3.154 Results of amlodipine standard at decreased organic solvent

No.	Ret. Time	Area	Tailing factor	Resolution
STD1	0.589	2495859	0.963	15.254
SDT2	0.595	2494525	0.96	15.299
STD3	0.593	2499501	0.962	15.285
STD4	0.591	2493669	0.963	15.263
STD5	0.585	2490348	0.962	15.24
STD6	0.592	2480490	0.963	15.219
Avg	**0.5912**	**2492399**	**0.962**	**15.2612**
STDEV	0.003768	6550.934	0.001225	0.032515
RSD	0.637397	0.262837	0.127312	0.213054

Table 3.155 Results of atorvastatine standard at decreased organic solvent

No.	Ret. Time	Area	Tailing factor	Resolution
STD1	4.976	2623723	1.134	15.254
SDT2	4.975	2627002	1.126	15.299
STD3	4.977	2627641	1.13	15.285
STD4	4.968	2630330	1.132	15.263
STD5	4.951	2616765	1.129	15.24
STD6	4.944	2612766	1.128	15.219
Avg	**4.963**	**2623038**	**1.129**	**15.2612**
STDEV	0.014748	6861.602	0.002236	0.032515
RSD	0.297157	0.26159	0.198057	0.213054

Table 3.156 Results of amlodipine and atorvastatine sample at increased organic solvent

	Amlodipine	atorvastatine
1st trial	2488751	2629077
2nd trial	2506273	2615988
3rd trial	2507322	2632173
Avg.	2500782	2625746
STDEV	10432.34	8591.288
RSD	0.417163	0.327194
Recovery %	**100.3364**	**100.1032**

Summary of recovery for both components at different conditions, average and RSD were shown in table 3.157 below.

Table 3.157 amlodipine and atorvastatine recovery at all robustness conditions

No	Condition	Amlodipine	Losartan
1	Optimized conditions	99.66346	99.94377278
2	Mor 5 degree Celsius	100.0107	99.79900504
3	less 5 degree Celsius	100.1828	99.75562597
4	5% More flow rate	99.83417	99.58700892
5	5% less flow rate	99.91241	99.37924743
6	5% more Organic solvent	100.3364	99.97884431
7	5% less Organic solvent	100.3895	99.84946469
	Avg.	**99.65178656**	**99.75613845**
	STDEV	**0.262766244**	**0.182937003**
	RSD %	**0.263684428**	**0.183384207**

3.3.7 Assay:

Standard solution and sample solution were prepared as described in section (2-4-3-11); standard solution was injected six times, while sample solution was injected three times, the average of each was used for assay calculations as shown in Table 3.158 and Table 3.159

Table 3.158 Results of mixed standard for assay

	Amlodipine	Atorvastatine
1	2508145	2529236
2	2528857	2530119
3	2514403	2535365
4	2490941	2527404
5	2498523	2490856
6	2486444	2493785
avg	**2504552**	**2517794**
STDEV	15812.61	19929.9
RSD	0.631355	0.791562

Table 3.159 Assay results for amlodipine and atorvastatine

	Amlodipine	Atorvastatine
1st trial	2490784	2511439
2nd trial	2490664	2533501
3rd trial	2505280	2504972
AVG	2495576	2516637
STDEV	8404.125	14958.04
RSD	0.336761	0.594366
Assay	**99.642**	**99.954**

3.4 Hydrochlorothiazide, Amlodipine besylate and Losartan Potassium

3.4.1 System Suitability

System suitability results for hydrochlorothiazide, amlodipine besylate and losartan potassium are shown in Table 3.160, Table 3.161 and Table 3.162, respectively.

Table 3.160 System suitability results for hydrochlorothiazide

No.	Retention time	Area	Theoretical plates	Tailing factor	Resolution
1	3.15	1284626	5903	1.422	8.85
2	3.15	1274131	5920	1.423	8.894
3	3.15	1279409	5953	1.422	8.927
4	3.148	1277206	5990	1.423	8.95
5	3.15	1279045	5968	1.428	8.965
6	3.152	1278067	5957	1.43	8.946
Avg	**3.15**	**1277571.6**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.001414214	2107.353743	25.461736	0.003563706	0.027300183
RSD	0.044895669	0.164949952	0.427382436	0.250049532	0.305494194

Table 3.161 System suitability results for Amlodipine besylate

No.	Retention time	Area	Theoretical plates	Tailing factor	Resolution
1	4.836	146064	7939	1.633	8.85
2	4.841	146875	7999	1.648	8.894
3	4.843	147677	8034	1.663	8.927
4	4.846	147013	8004	1.654	8.95
5	4.85	147166	8054	1.662	8.965
6	4.855	148323	7982	1.661	8.946
Avg	**4.847**	**147410.8**	**8014.6**	**1.6576**	**8.9364**
STDEV	0.005612486	593.3432396	28.92749557	0.006426508	0.027300183
RSD	0.115792987	0.402510019	0.360934988	0.387699542	0.305494194

Table 3.162 System suitability results for Losartan Potassium

No.	Retention time	Area	Theoretical plates	Tailing factor	Resolution
1	5.943	2947909	10706	1.357	4.956
2	5.946	2948752	10756	1.363	4.959
3	5.947	2948303	10806	1.365	4.962
4	5.949	2948774	10785	1.367	4.948
5	5.952	2948493	10790	1.369	4.948
6	5.954	2948280	10752	1.372	4.919
Avg	**5.9496**	**2948520.4**	**10777.8**	**1.3672**	**4.9472**
STDEV	0.003361547	236.514906	23.11276703	0.00349285	0.016991174
RSD	0.056500391	0.008021478	0.214447912	0.255474681	0.343450319

3.4.2 Linearity, LOD and LOQ

i) Hydrochlorothiazide

Table 3.163 show linearity results for hydrochlorothiazide which then treated by XLSTAT-2015 program to predict linearity data that shown in Table 3.164, Table 3.165, Figure 3.22 and Figure 3.23.

Table 3.163 linearity result for hydrochlorothiazide

Column1	40	60	80	100	120	140	160
1	513443	769401	1033308	1270602	1522206	1777958	2041040
2	512589	769361	1033091	1272361	1523055	1772431	2038883
3	512201	769218	1029487	1278668	1521097	1777751	2053000
avg	**512744.33**	**769326.667**	**1031962**	**1273877**	**1522119.333**	**1776046.67**	**2044307.667**
STDEV	635.40328	96.20984032	2146.1573	4241.31831	981.8728703	3132.969252	7604.647022
RSD	0.123922	0.01250572	0.2079686	0.33294567	0.064506957	0.176401291	0.371991317

Figure 3.22 shows the plot of average area versus concentrations for hydrochlorothiazide in µg/ml, the linear regression equation:

$$\text{Area} = 8217.79+50702.05*\mu g/ml$$

According to ICH guidelines, acceptance criteria is $R^2 \geq 0.997$.

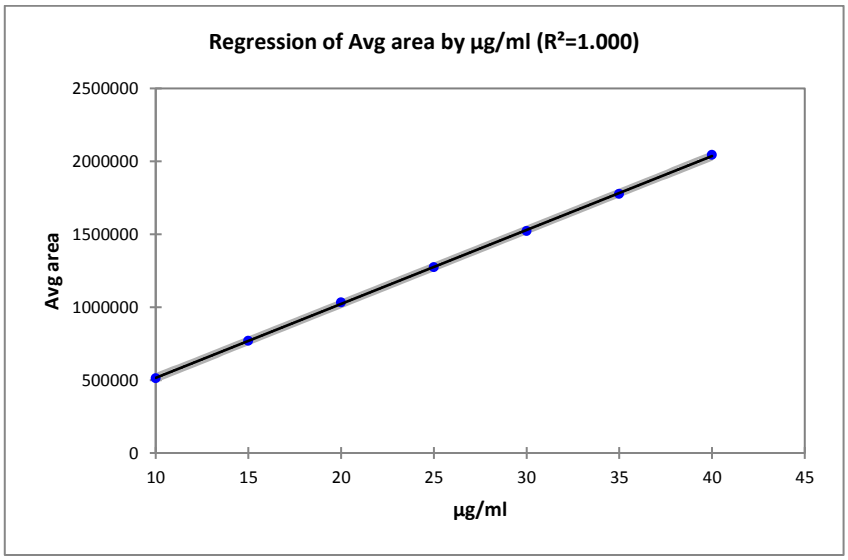

Figure 3.22 XL STAT 2015 plot of conc. in µg/ml vs average area of hydrochlorothiazide

Table 3.164 XL- STAT 2015 Goodness of fit statistics for hydrochlorothiazide

Goodness of fit statistics:	
Observations	7.000
Sum of weights	7.000
R^2	1.000
Adjusted R^2	1.000
MSE	53028711.781
RMSE	7282.082

Table 3.165 XL STAT 2015 predicted area for hydrochlorothiazide

Observation	Weight	µg/ml	Average area	Predicted area
Obs1	1	10.000	512744.333	515238.310
Obs2	1	15.000	769326.667	768748.571
Obs3	1	20.000	1031962.000	1022258.833
Obs4	1	25.000	1273877.000	1275769.095
Obs5	1	30.000	1522119.333	1529279.357
Obs6	1	35.000	1776046.667	1782789.619
Obs7	1	40.000	2044307.667	2036299.881

Figure 3.23 is the a plot of average area versus predicted area for hydrochlorothiazide , i.e. concentration versus predicted concentration of hydrochlorothiazide, acceptance limit for this graph is that slope ≥ 0.997

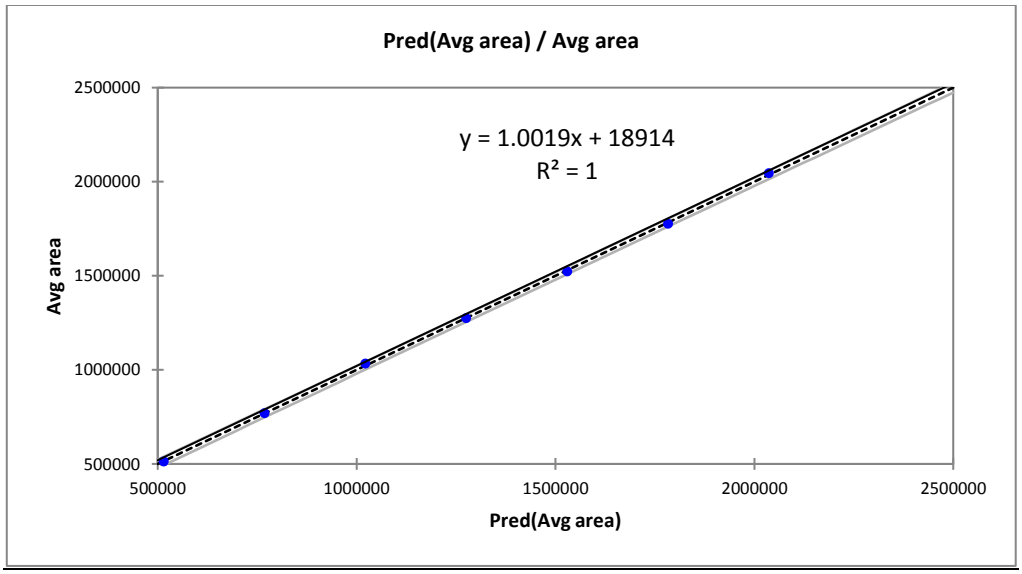

Figure 3.16 XL- STAT 2015 plot of area Vs Predicted area for hydrochlorothiazide

Limit of Detection and Quantitation

LOD = 3.3* (SD/S).

LOD = 3.3* (7282/50702) = **0.47µg/ml**

LOD % (relative to target concentration) = 0.47*100/25 = 1.88%

LOQ = 10 * (SD/S).

LOQ = 10* (7282/50702)= **1.44µg/ml**

LOQ % (relative to target concentration) = 1.44*100/25 = 5.76%

ii) Amlodipine besylate

Table 3.166 show linearity results for amlodipine besylate which then treated by XLSTAT-2015 program to predict linearity data that shown in Table 3.167, Table 3.168, Figure 3.24 and Figure 3.25.

Table 3.166 linearity result for amlodipine besylate

Content	40	60	80	100	120	140	160
1	57976	87602	119742	147481	177357	205683	235641
2	58063	87440	118782	147869	176770	205348	234666
3	59010	87171	117327	147967	176304	205815	234573
avg	**58349.667**	**87404.3333**	**118617**	**147772.33**	**176810.3333**	**205615.333**	**234960**
STDEV	573.51751	217.7023963	1215.9256	257.016212	527.6574015	240.7412165	591.5936105
RSD	0.9828977	0.249075061	1.0250854	0.17392715	0.298431314	0.117083299	0.251784819

Figure 3.24 shows the plot of average area versus concentrations for amlodipine besylate in µg/ml, the linear regression equation:

Area = -146.99+14722.26*µg/ml

According to ICH guidelines, acceptance criteria is $R^2 \geq 0.997$.

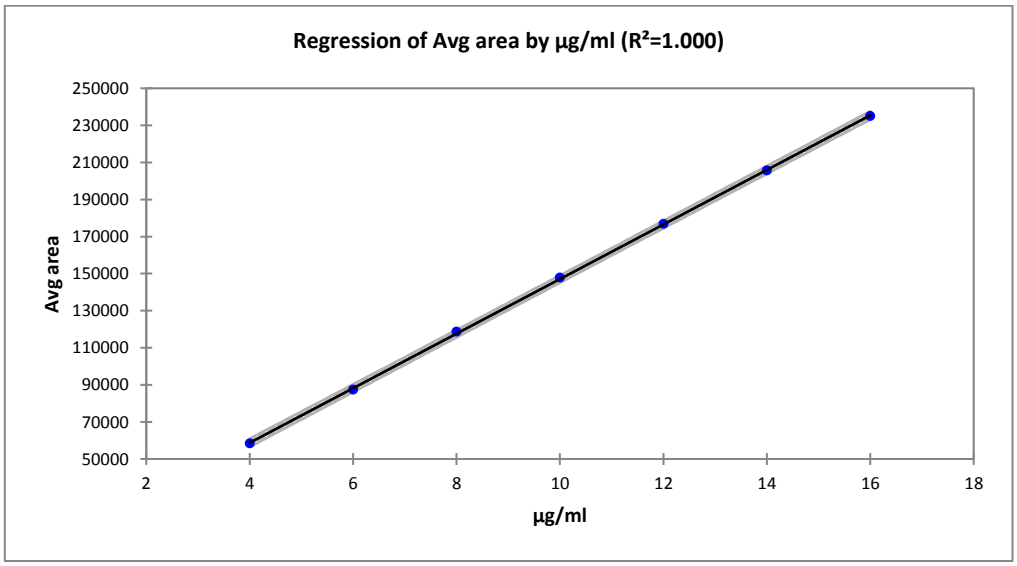

Figure 3.24 XL STAT 2015 plot of conc. in µg/ml vs average area of amlodipine besylate

Table 3.167 XL- STAT 2015 Goodness of fit statistics for amlodipine besylate

Goodness of fit statistics:	
Observations	7.000
Sum of weights	7.000
R^2	1.000
Adjusted R^2	1.000
MSE	526258.920
RMSE	725.437

Table 3.168 XL STAT 2015 predicted area for amlodipine besylate

Observation	Weight	µg/ml	Avg area	Pred(Avg area)
Obs1	1	4.000	58349.667	58742.036
Obs2	1	6.000	87404.333	88186.548
Obs3	1	8.000	118617.000	117631.060
Obs4	1	10.000	147772.333	147075.571
Obs5	1	12.000	176810.333	176520.083
Obs6	1	14.000	205615.333	205964.595
Obs7	1	16.000	234960.000	235409.107

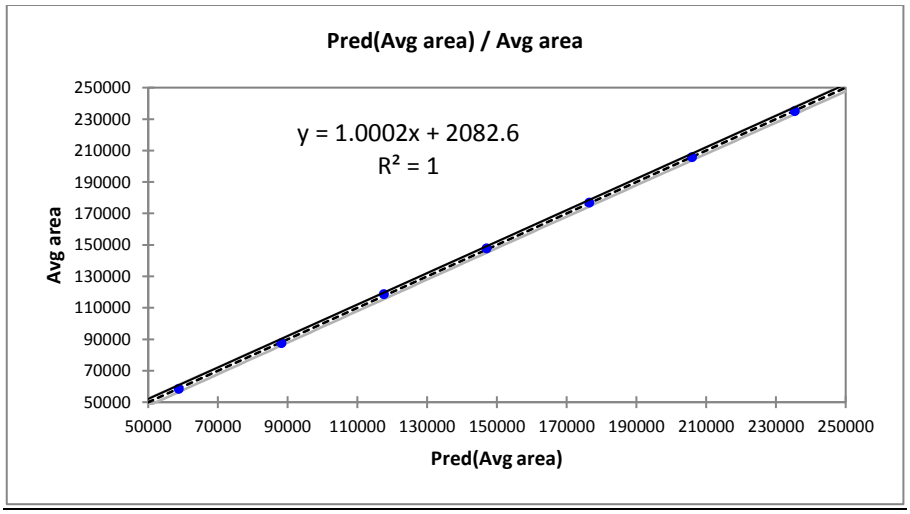

Figure 3.25 Plot of average area versus predicted area for amlodipine acceptance limit for this graph is that slope ≥ 0.997

Limit of detection and limit of quantitation

LOD = 3.3* (SD/S).

LOD = 3.3* (725/14722) = **0.16 µg/ml**

LOD % (relative to target concentration) = 0.16*100/10 = 1.6%

LOQ = 10 * (SD/S).

LOQ = 10* (725/14722)

LOQ = = **0.49 µg/ml**

LOQ % (relative to target concentration) = 0.49*100/10 = 4.9%

iii) Losartan potassium

Table 3.169 show linearity results for losartan potassium which was then treated by XLSTAT-2015 program to predict linearity data that shown in Table 3.170, Table 3.171, Figure 3.26 and Figure 3.27.

Table 3.169 linearity result for losartan potassium

Conc µg/ml	Content	40	60	80	100	120	140
1	1	1158773	1748272	2341694	2938140	3552978	4129232
2	2	1163346	1756893	2353156	2937679	3525086	4131245
3	3	1158690	1755922	2354810	2937674	3539002	4116482
avg	avg	**1160269.7**	**1753695.67**	**2349887**	**2937831**	**3539022**	**4125653**
STDEV	STDEV	2664.506	4722.057849	7143.0924	267.613527	13946.01076	8005.839931
RSD	RSD	0.2296454	0.269263244	0.303976	0.00910922	0.394063975	0.194050249

Figure 3.26 shows the plot of average area versus concentrations for losartan potassium in µg/ml, the linear regression equation:

$$\text{Area} = -18469.24 + 29567.98 * \mu g/ml$$

According to ICH guidelines, acceptance criteria is $R^2 \geq 0.997$.

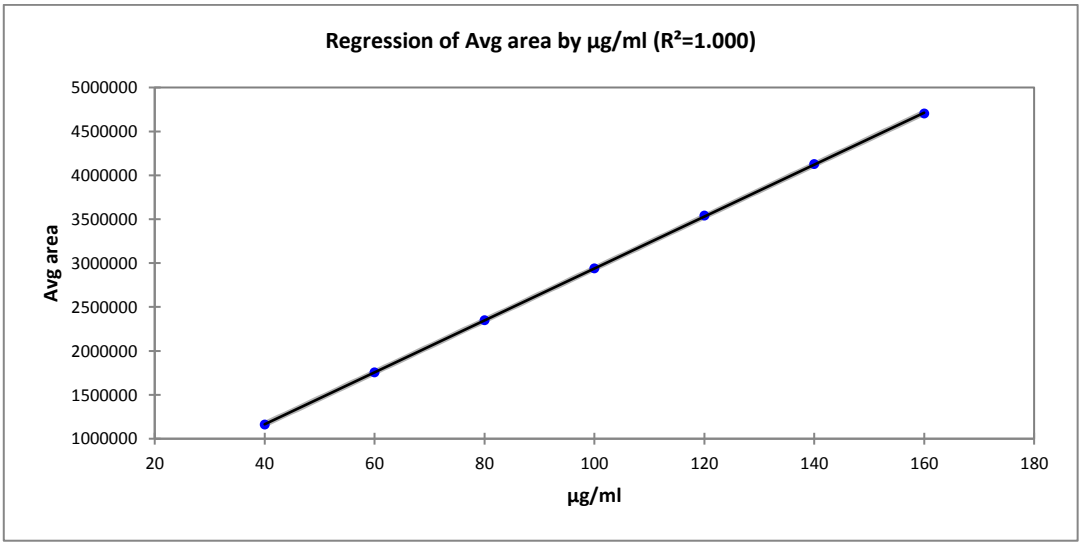

Figure 3.26 XL STAT 2015 plot of conc. in µg/ml vs average area of losartan potassium

Table 3.170 XL- STAT 2015 Goodness of fit statistics for losartan potassium

Goodness of fit statistics:	
Observations	7.000
Sum of weights	7.000
R^2	1.000
Adjusted R^2	1.000
MSE	49221906.190
RMSE	7015.833

Table 3.171 XL STAT 2015 predicted area for losartan potassium

Observation	Weight	µg/ml	Avg area	Pred(Avg area)
Obs1	1	40.000	1160269.667	1164249.905
Obs2	1	60.000	1753695.667	1755609.476
Obs3	1	80.000	2349886.667	2346969.048
Obs4	1	100.000	2937831.000	2938328.619
Obs5	1	120.000	3539022.000	3529688.190
Obs6	1	140.000	4125653.000	4121047.762
Obs7	1	160.000	4701942.333	4712407.333

Figure 3.27 is the a plot of average area versus predicted area for losartan potassium , i.e. concentration Vs predicted concentration of losartan potassium, acceptance limit for this graph is that slope ≥ 0.997

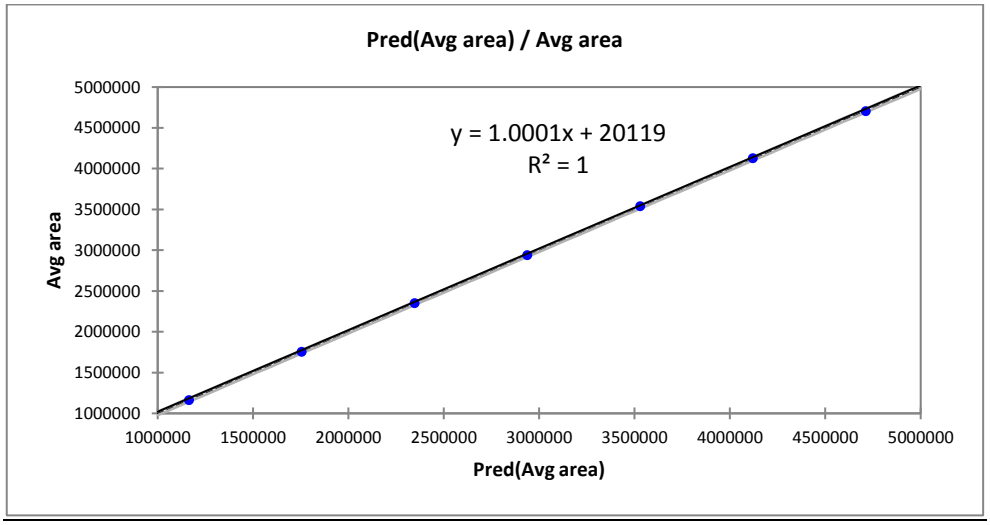

Figure 3.27 XL- STAT 2015 plot of area Vs Predicted area for losartan potassium

Limit of Detection and Quantitation

LOD = 3.3* (SD/S).

LOD = 3.3* (7015/29568) = **0.78 µg/ml**

LOD % (relative to target concentration) = 078*100/100 = 3.7%

LOQ = 10 * (SD/S).

LOQ = 10* (24414/141913)

LOQ = **2.37 µg/ml**

LOQ % (relative to target concentration) = 2.37*100/100 = 2.37%

3.4.3 Specificity

Figure 3.28, Figure 3.29 and Figure 3.30 shows the specificity chromatograms for placebo, sample and standard respectively for hydrochlorothiazide, amlodipine besylate and losartan potassium.

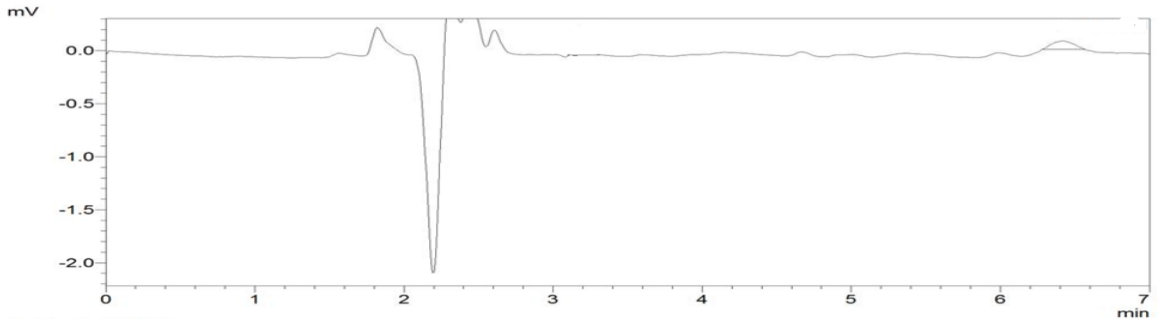

Figure 3.28 Chromatogram for the Placebo of hydrochlorothiazide, amlodipine besylate and losartan potassium.

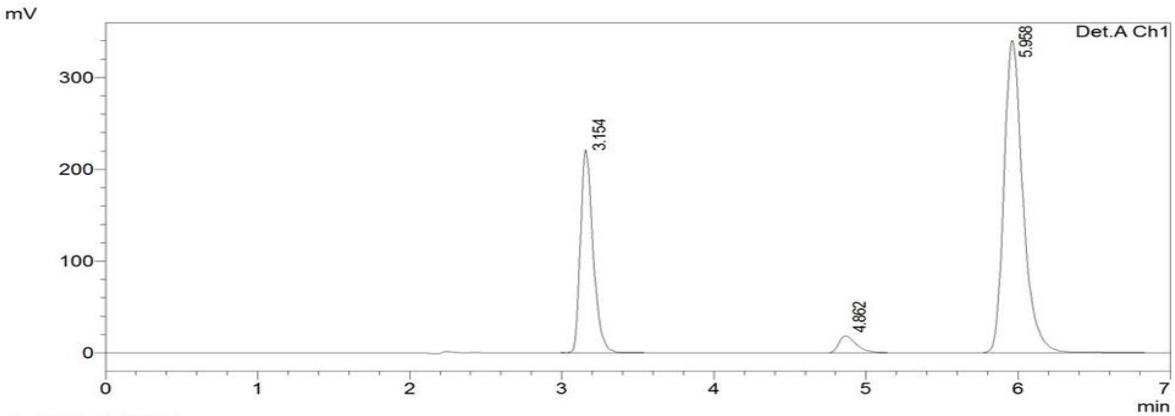

figure 3.29 chromatogram for the sample of hydrochlorothiazide, amlodipine besylate and losartan potassium.

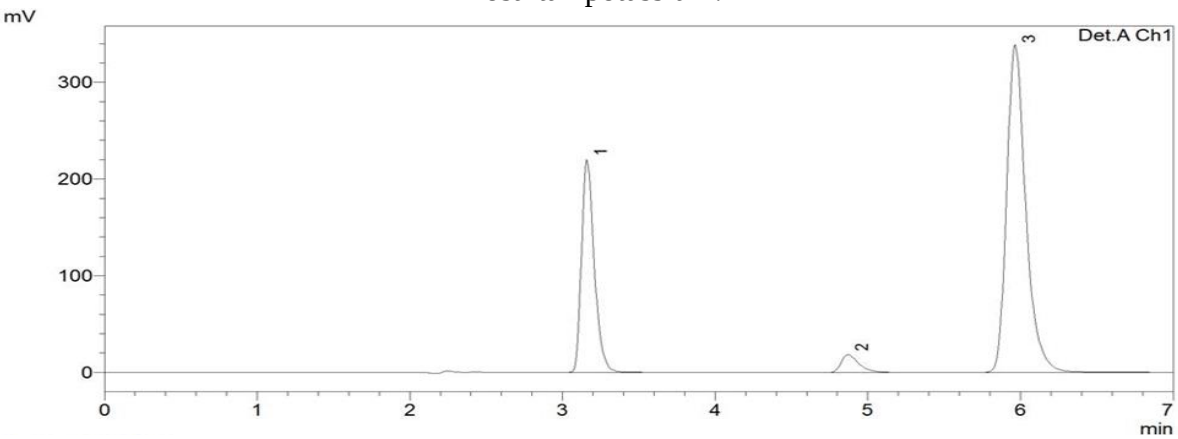

figure 3.30 chromatogram for mixed standard of hydrochlorothiazide, amlodipine besylate and losartan potassium.

3.4.4 Accuracy

Table 3.172 shows the results of mixed standard of hydrochlorothiazide, amlodipine besylate and losartan potassium, while the accuracy results for samples are shown in Table 3.173, Table 3.174 and Table 3.175, respectively; summary of accuracy results for the triple mixture is shown in Table 3.176.

Table 3.172 hydrochlorothiazide, amlodipine and losartan standard for accuracy test

No.	Hydrochlorothiazide	amlodipine besylate	losartan potassium
STD1	1284626	146064	2947909
SDT2	1274131	146875	2948752
STD3	1279409	147677	2948303
STD4	1277206	147013	2948774
STD5	1279045	147166	2948493
STD6	1278067	148323	2948280
Avg	**1277571.6**	**147410.8**	**2948520**
STDEV	2107.3537	593.34324	236.5149
RSD	0.16495	0.40251	0.008021

Table 3.173 Accuracy results for hydrochlorothiazide

Content	40	60	80	100	120	140	160
1	515258	768726	1033365	1274234	1532701	1780695	2015245
2	516464	768021	1034694	1274455	1536387	1775980	2020309
3	513961	766775	1033611	1273943	1533024	1776747	2020526
avg	515227.67	767840.6667	1033890	1274211	1534037	1777807	2018693
STDEV	1251.7757	987.9222304	707.0651	256.7963	2041.27	2530.027	2988.315
RSD	0.2429558	0.1286624	0.068389	0.020153	0.133065	0.142312	0.148032
RECOVERY	**40.328673**	**60.10157604**	**80.92619**	**99.73693**	**120.0745**	**139.1552**	**158.0102**
RECOVERY %	**100.82168**	**100.1692934**	**101.1577**	**99.73693**	**100.0621**	**99.39657**	**98.75637**

Table 3.174 Accuracy results for amlodipine

Content	40	60	80	100	120	140	160
1	58163	88096	118889	147221	177219	2E+05	234560
2	58560	87275	118701	146235	177671	2E+05	235385
3	58554	87969	118522	147150	177482	2E+05	235286
avg	58426	87780	118704	146869	177457	2E+05	235077
STDEV	227.5	441.929	183.52	549.92	227.01	263.8	450.46
RSD	0.3894	0.50345	0.1546	0.3744	0.1279	0.128	0.1916
RECOVERY	**39.635**	**59.5479**	**80.526**	**99.632**	**120.38**	**140**	**159.47**
RECOVERY %	**99.086**	**99.2465**	**100.66**	**99.632**	**100.32**	**99.97**	**99.669**

Table 3.175 Accuracy results for losartan

Content	40	60	80	100	120	140	160
1	1154734	1757825	2330412	2933478	3577435	4150478	4722456
2	1158750	1768098	2331832	2935727	3542316	4134893	4715734
3	1155085	1767556	2330854	2935304	3560167	4133893	4748636
avg	1156189.7	1764493	2331033	2934836	3559973	4139755	4728942
STDEV	2224.2483	5781.012801	726.6645	1195.213	17560.31	9300.129	17383.51
RSD	0.1923775	0.327630248	0.031174	0.040725	0.493271	0.224654	0.367598
RECOVERY	**39.212543**	**59.84334514**	**81.83312**	**99.53591**	**120.7376**	**140.4011**	**160.3836**
RECOVERY %	**98.031357**	**99.73890856**	**102.2914**	**99.53591**	**100.6147**	**100.2865**	**100.2397**

Table 3.176 Summary of accuracy results for hydrochlorothiazide, amlodipine and losartan

Content%	hydrochlorothiazide	amlodipine	losartan
40	100.8216813	99.0865	98.0314
60	100.1692934	99.2465	99.7389
80	101.1577355	100.657	102.291
100	99.73692799	99.6322	99.5359
120	100.0620587	100.319	100.615
140	99.39657245	99.968	100.287
160	98.75636977	99.6692	100.24
avg	99.40500031	99.9854	100.38145
STDEV	0.65288527	0.32529	0.20431145
RSD	0.656793187	0.32534	0.20354

3.4.5 Precision

i) Intraday Precision

Table 3.177 show results of hydrochlorothiazide, amlodipine besylate and losartan potassium mixed standard for intraday precision test.

Table 3.177 hydrochlorothiazide, amlodipine and losartan standard for intraday precision

No.	hydrochlorothiazide	amlodipine	losartan
STD1	1284626	146064	2607554
SDT2	1274131	146875	2632440
STD3	1279409	147677	2635480
STD4	1277206	147013	2654284
STD5	1279045	147166	2594729
STD6	1278067	148323	2599983
Avg	**1277571.6**	**147410.8**	**2620745**
STDEV	2107.353743	593.3432396	23497.46
RSD	0.164949952	0.402510019	**0.896595**

For intraday precision of mixed solutions containing 80%, 100% and 120% from each component, tables numbered 3.178, 3.179 and 3.180 show results of hydrochlorothiazide, respectively; tables numbered 3.181, 3.182 and 3.183 show results for amlodipine, respectively; while tables numbered 3.184, 3.185 and 3.186 show intraday precision results for losartan. Table 3.196 show the summary of the previous nine tables, the average and RSD of each five assays of the three concentrations for each active ingredient.

Table 3.178 Intraday results for 80% hydrochlorothiazide

	1st	2nd	3rd	4th	5th
1st trial	1031415	1031491	1030537	1033153	1032776
2nd trial	1032874	1033506	1037127	1033537	1037295
3rd trial	1031490	1032142	1033603	1033786	1036237
AVG	1031926	1032380	1033756	1033492	1035436
STDEV	821.5597	1028.309	3297.65	318.89	2363.59
RSD	0.079614	0.099606	0.319	0.03086	0.22827
RECOVERY	**80.7725**	**80.808**	**80.916**	**80.895**	**81.047**
RECOVERY %	**100.966**	**101.01**	**101.14**	**101.12**	**101.31**

Table 3.179 Intraday results for 100% hydrochlorothiazide

	1st	2nd	3rd	4th	5th
1st trial	1275387	1282957	1283261	1284150	1284472
2nd trial	1281560	1285025	1283692	1282561	1286355
3rd trial	1282914	1282891	1281974	1280121	1287593
AVG	1279954	1283624	1282976	1282277	1286140
STDEV	4012.376	1213.462	893.836	2029.42	1571.57
RSD	0.313478	0.094534	0.06967	0.15827	0.12219
RECOVERY	**100.186**	**100.474**	**100.42**	**100.37**	**100.67**
RECOVERY %	**100.186**	**100.474**	**100.42**	**100.37**	**100.67**

Table 3.180 Intraday results for 120% hydrochlorothiazide

	1st	2nd	3rd	4th	5th
1st trial	1532727	1534799	1536792	1534423	1535910
2nd trial	1529075	1535772	1536634	1534371	1537497
3rd trial	1535704	1534946	1534925	1537072	1535492
AVG	1535704	1535172	1536117	1535289	1536300
STDEV	3320.223	524.502	1035.32	1544.63	1057.77
RSD	0.216202	0.034166	0.0674	0.10061	0.06885
RECOVERY	**120.205**	**120.163**	**120.24**	**120.17**	**120.25**
RECOVERY %	**100.171**	**100.136**	**100.2**	**100.14**	**100.21**

Table 3.181 Intraday results for 80% amlodipine

	1st	2nd	3rd	4th	5th
1st trial	118921	119387	119262	119247	118770
2nd trial	119345	119793	119331	119601	118854
3rd trial	119742	119853	119424	119620	118917
AVG	119336	119677.7	119339	119489	118847
STDEV	410.574	253.5061	81.2958	210.082	73.7496
RSD	0.344049	0.211824	0.06812	0.17582	0.06205
RECOVERY	**80.9547**	**81.1865**	**80.957**	**81.059**	**80.623**
RECOVERY %	**101.193**	**101.483**	**101.2**	**101.32**	**100.78**

Table 3.182 Intraday results for 100% amlodipine

	1st	2nd	3rd	4th	5th
1st trial	148481	148766	148641	149004	147916
2nd trial	149052	149193	148613	148583	147680
3rd trial	148980	149226	149115	148231	148216
AVG	148837.7	149061.7	148790	148606	147937
STDEV	310.9732	256.5859	282.095	387.013	268.636
RSD	0.208934	0.172134	0.18959	0.26043	0.18159
RECOVERY	**100.968**	**101.12**	**100.94**	**100.81**	**100.36**
RECOVERY %	**100.968**	**101.12**	**100.94**	**100.81**	**100.36**

Table 3.183 Intraday results for 120% amlodipine

	1st	2nd	3rd	4th	5th
1st trial	178287	178466	178654	178067	177948
2nd trial	178336	178727	178549	178022	177853
3rd trial	178545	178146	178711	177737	177485
AVG	178389.3	178446.3	178638	177942	177762
STDEV	137.0195	290.9989	82.1766	178.955	244.547
RSD	0.076809	0.163074	0.046	0.10057	0.13757
RECOVERY	**121.015**	**121.054**	**121.18**	**120.71**	**120.59**
RECOVERY %	**100.846**	**100.878**	**100.99**	**100.59**	**100.49**

Table 3.184 Intraday results for 80% losartan

	1st	2nd	3rd	4th	5th
1st trial	2330914	2337089	2332020	2333051	2329855
2nd trial	2333191	2333436	2334033	2333799	2331519
3rd trial	2330504	2332652	2334034	2335644	2332216
AVG	2331536	2334392	2333362	2334165	2331197
STDEV	1447.573	2368.052	1162.49	1334.61	1213.06
RSD	0.062087	0.101442	0.04982	0.05718	0.05204
RECOVERY	**79.0748**	**79.1717**	**79.137**	**79.164**	**79.063**
RECOVERY %	**98.8435**	**98.9646**	**98.921**	**98.955**	**98.829**

Table 3.185 Intraday results for 100% losartan

	1st	2nd	3rd	4th	5th
1st trial	2936335	2953940	2952077	2952237	2952023
2nd trial	2955309	2956932	2954047	2952712	2951640
3rd trial	2953149	2955845	2955845	2951280	2950698
AVG	2948264	2955572	2953990	2952076	2951454
STDEV	10387.4	1514.522	1884.65	729.394	681.87
RSD	0.352323	0.051243	0.0638	0.02471	0.0231
RECOVERY	**99.9913**	**100.239**	**100.19**	**100.12**	**100.1**
RECOVERY %	**99.9913**	**100.239**	**100.19**	**100.12**	**100.1**

Table 3.186 Intraday results for 120% losartan

	1st	2nd	3rd	4th	5th
1st trial	3569079	3560943	3573877	3567272	3565628
2nd trial	3565659	3576463	3555406	3562766	3579266
3rd trial	3556778	3571013	3563576	3558474	3575839
AVG	3563839	3569473	3564286	3562837	3573578
STDEV	6349.32	7873.773	9255.97	4399.43	7094.64
RSD	0.17816	0.220586	0.25969	0.12348	0.19853
RECOVERY	**120.869**	**121.06**	**120.88**	**120.83**	**121.2**
RECOVERY %	**100.724**	**100.883**	**100.74**	**100.7**	**101**

Table 3.187 Summery of intraday precision for hydrochlorothiazide, amlodipine and losartan

	hydrochlorothiazide			amlodipine			losartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
STD1	80 %	100 %	120 %	80 %	100 %	120 %	80 %	100 %	120 %
SDT2	100.97	100.19	100.17	101.19	100.97	100.85	98.843	99.991	100.72
STD3	101.01	100.47	100.14	101.48	101.12	100.88	98.965	100.24	100.88
STD4	101.14	100.42	100.2	101.2	100.94	100.99	98.921	100.19	100.74
STD5	101.12	100.37	100.14	101.32	100.81	100.59	98.955	100.12	100.7
STD6	101.31	100.67	100.21	100.78	100.36	100.49	98.829	100.1	101
Avg.	**101.11**	**100.42**	**100.17**	**101.19**	**100.84**	**100.76**	**98.903**	**100.13**	**100.81**
STDEV	0.1339	0.1753	0.0323	0.2611	0.2906	0.2078	0.0629	0.0938	0.1294
RSD	**0.1324**	**0.1745**	**0.0322**	**0.2581**	**0.2882**	**0.2062**	**0.0636**	**0.0937**	**0.1284**

ii) Interday Precision

Table 3.188 show results of hydrochlorothiazide, amlodipine and losartan mixed standard for interday precision test. Tables numbered 3.189, 3.190 and 3.191 show intraday precision for 80%, 100% and 120% for the three components, respectively.Table 3.192 show the summary of interday precision, the average and RSD of each three assays of the three concentrations for each active ingredient.

Table 3.188 hydrochlorothiazide, amlodipine and losartan standard for interday precision

	hydrochlorothiazide			amlodipine			losartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
STD1	1284626	1285386	1282828	146064	147923	147744	2947909	2944787	2949409
SDT2	1274131	1282487	1282373	146875	147708	147744	2948752	2948452	2948009
STD3	1279409	1304488	1280749	147677	148008	147733	2948303	2948618	2947858
STD4	1277206	1281264	1286172	147013	147612	147605	2948774	2948602	2948915
STD5	1279045	1279833	1281993	147166	148053	147784	2948493	2948437	2949006
STD6	1278067	1281111	1288062	148323	148060	147882	2948280	2948576	2948802
Avg.	**1277571.6**	**1285836.6**	**1283869.8**	**147410.8**	**147888.2**	**147749.6**	**2948520.4**	**2948537**	**2948518**
STDEV	2107.35	10468.73	3097.45	593.34	212.005	99.911	236.51	85.924	541.08
RSD	0.165	0.814	0.24126	0.4025	0.143	0.0676	0.00802	0.0029	0.018

Table 3.189 Interday precision results for 80% hydrochlorothiazide, amlodipine and losartan

	hydrochlorothiazide			amlodipine			losartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
assay 1	1033365	1033718	1031064	118889	118496	118798	2330412	2335243	2336841
assay 2	1034694	1034144	1035399	118701	119181	119081	2331832	2335840	2338653
assay 3	1033611	1031832	1039425	118522	119171	118993	2338054	2336594	2338498
Avg	1033890	1033231.3	1035296	118704	118949.33	118957.33	2333432.7	2335892.3	2337997
STDEV	707.065	1230.43	4181.45	183.51839	392.63002	144.83209	4064.6822	677.019	1004.41
RSD	0.06838	0.119	0.4039	0.1546017	0.3300817	0.1217513	0.1741932	0.02898	0.04296
Recovery	80.9261	80.3548	80.6387	80.525986	80.43193	80.512796	79.139105	79.222	79.294
Recovery%	101.157	100.443	100.798	100.65748	100.53991	100.64099	98.923882	99.0276	99.1175

Table 3.190 Interday precision results for 100% hydrochlorothiazide, amlodipine and losartan

	hydrochlorothiazide			amlodipine			losartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
assay 1	1274234	1285079	1284254	147221	148199	148481	2933478	2956620	2960910
assay 2	1274455	1283645	1286359	146235	148192	148585	2935727	2957201	2964015
assay 3	1273943	1281939	1286330	147150	148243	148489	2935304	2957707	2963609
Avg	1274210.7	1283554.3	1285647.7	146868.67	148211.33	148518	2934836.3	2957176	2962845
STDEV	256.79629	1571.9623	1207.0378	549.91848	27.646579	57.8734	1195.2131	543.93106	1687.72
RSD	0.0201534	0.1224695	0.0938856	0.3744287	0.0186535	0.03897	0.040725	0.0183936	0.05696
Recovery	99.736928	99.822507	100.13848	99.63223	100.2185	100.52	99.535901	100.29299	100.486
Recovery%	99.736928	99.822507	100.13848	99.63223	100.2185	100.52	99.535901	100.29299	100.486

Table 3.191 interday precision results for 120% hydrochlorothiazide, amlodipine and losartan

	hydrochlorothiazide			amlodipine			losartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
assay 1	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
assay 2	1532701	1535178	1537188	177219	177801	178291	3569079	3578879	3572896
assay 3	1536387	1538125	1538155	177671	177938	178235	3565659	3565750	3563545
Avg	1533024	1531323	1534509	177482	178223	178287	3556778	3560488	3567915
STDEV	1534037.3	1534875.3	1536617.3	177457.33	177987.33	178271	3563838.7	3568372.3	3568118.6
RSD	2041.2698	3411.0858	1888.8024	227.00734	215.28199	31.241	6349.32	9471.78	4678.83
Recovery	0.1330652	0.2222386	0.1229195	0.1279222	0.1209535	0.01752	0.178	0.2654	0.13
Recovery%	120.07447	119.36784	119.68638	120.38286	120.35263	120.658	**120.87**	121.02	121.01

Table 3.192 interday precision summery for hydrochlorothiazide, amlodipine and losartan

	hydrochlorothiazide			amlodipine			losartan		
	80%	100%	120%	80%	100%	120%	80%	100%	120%
Day 1	101.16	99.737	100.06	100.66	99.632	100.32	98.924	99.536	100.7239
Day 2	100.44	99.823	99.473	100.54	100.22	100.29	99.028	100.29	100.8515
Day 3	100.8	100.14	99.739	100.64	100.52	100.55	99.117	100.49	100.845
Avg	100.8	99.899	99.758	100.61	100.12	100.39	99.023	100.1	100.8068
STDEV	0.3571	0.2115	0.2949	0.0637	0.4516	0.14	0.0969	0.5021	0.071834
RSD	0.3543	0.2117	0.2956	0.0633	0.451	0.1394	0.0978	0.5016	0.07126

3.4.6 Robustness

The method was examined for robustness test under nine different conditions comparing the method output under each condition with that of the optimized conditions and with permissible limits according to ICH. Lastly the variation in method output was evaluated through calculation of the average and RSD% of the nine results obtained under the different nine conditions, detailed results are shown in the followings.

i) Optimized conditions

Standard solution was injected six times while sample solution was injected three times under optimized conditions. Results hydrochlorothiazide, amlodipine and losartan standards are shown in Table 3.193, Table 3.194 and Table 3.195, respectively; results of samples for the three components are shown in Table 3.196.

Table 3.136 Table 3.193 Results of hydrochlorothiazide standard at optimum conditions

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	1284626	5903	1.422	8.85
SDT2	3.15	1274131	5920	1.423	8.894
STD3	3.15	1279409	5953	1.422	8.927
STD4	3.148	1277206	5990	1.423	8.95
STD5	3.15	1279045	5968	1.428	8.965
STD6	3.152	1278067	5957	1.43	8.946
Avg	**3.15**	**1277571.6**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	2107.35374	25.461736	0.003564	0.0273
RSD	0.0448957	0.16494995	0.4273824	0.25005	0.305494

Table 3.194 Results of amlodipine standard at optimum conditions

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	4.836	146064	7939	1.633	8.85
SDT2	4.841	146875	7999	1.648	8.894
STD3	4.843	147677	8034	1.663	8.927
STD4	4.846	147013	8004	1.654	8.95
STD5	4.85	147166	8054	1.662	8.965
STD6	4.855	148323	7982	1.661	8.946
Avg	**4.847**	**147410.8**	**8014.6**	**1.6576**	**8.9364**
STDEV	0.005612486	593.3432396	28.92749557	0.006426508	0.027300183
RSD	0.115792987	0.402510019	0.360934988	0.387699542	0.305494194

Table 3.195 Results of losartan standard at optimum conditions

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	2947909	5903	1.422	8.85
SDT2	3.15	2948752	5920	1.423	8.894
STD3	3.15	2948303	5953	1.422	8.927
STD4	3.148	2948774	5990	1.423	8.95
STD5	3.15	2948493	5968	1.428	8.965
STD6	3.152	2948280	5957	1.43	8.946
Avg	**3.15**	**2948520.4**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	**236.514906**	25.461736	0.003564	0.0273
RSD	0.0448957	**0.00802148**	0.4273824	0.25005	0.305494

Table 3.196 Results of hydrochlorothiazide, amlodipine and losartan sample at optimum conditions

	hydrochlorothiazide	amlodipine	losartan
1st trial	1274234	147221	197093
2nd trial	1274455	146235	194216
3rd trial	1273943	147150	194269
Avg	1274210.67	146868.667	195192.667
STDEV	256.796288	549.918479	1645.95028
RSD	0.02015336	0.37442873	0.84324391
Recov%	**99.736928**	**99.6322296**	**99.84782**

ii) 5^{o}C more

Standard solution was injected six times while sample solution was injected three times after the column temperature was raised up five degrees celsius, Results of hydrochlorothiazide, amlodipine and losartan standards are shown in Table 3.197, Table 3.198 and Table 3.199, respectively; results of samples for the three components are shown in table 3.200.

Table 3.197 Results of hydrochlorothiazide standard at increased temperature

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	1282397	5903	1.422	8.85
SDT2	3.15	1281905	5920	1.423	8.894
STD3	3.15	1281126	5953	1.422	8.927
STD4	3.148	1280263	5990	1.423	8.95
STD5	3.15	1281075	5968	1.428	8.965
STD6	3.152	1283143	5957	1.43	8.946
Avg	**3.15**	**1281651.5**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	1037.40441	25.461736	0.003564	0.0273
RSD	0.0448957	0.08094278	0.4273824	0.25005	0.305494

Table 3.198 Results of amlodipine standard at increased temperature

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	148107	5903	1.422	8.85
SDT2	3.15	148047	5920	1.423	8.894
STD3	3.15	148127	5953	1.422	8.927
STD4	3.148	149922	5990	1.423	8.95
STD5	3.15	147988	5968	1.428	8.965
STD6	3.152	148265	5957	1.43	8.946
Avg	**3.15**	**148409.333**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	746.849025	25.461736	0.003564	0.0273
RSD	0.0448957	0.50323589	0.4273824	0.25005	0.305494

Table 3.199 Results of losartan standard at increased temperature

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	2956056	5903	1.422	8.85
SDT2	3.15	2950750	5920	1.423	8.894
STD3	3.15	2950599	5953	1.422	8.927
STD4	3.148	2951165	5990	1.423	8.95
STD5	3.15	2951667	5968	1.428	8.965
STD6	3.152	2951298	5957	1.43	8.946
Avg	**3.15**	**2951922.5**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	**2061.13161**	25.461736	0.003564	0.0273
RSD	0.0448957	**0.06982336**	0.4273824	0.25005	0.305494

Table 3.200 Results of hydrochlorothiazide, amlodipine and losartan sample at increased temperature

	hydrochlorothiazide	amlodipine	losartan
1st trial	1288052	148773	2958814
2nd trial	1287563	148900	2956748
3rd trial	1282732	148789	2956748
Avg	1286115.67	148820.667	**2957436.67**
STDEV	2940.52382	69.1688755	**1192.80566**
RSD	0.22863603	0.046478	**0.040332**
Recov%	**100.3483**	**100.2772**	**100.1868**

iii) 5°C less

Standard solution was injected six times while sample solution was injected three times after the column temperature was decreased five celsius degrees . Results of hydrochlorothiazide, amlodipine and losartan standards are shown in Table 3.201, Table 3.202 and Table 3.203, respectively; results of samples for the three components, are shown in Table 3.204.

Table 3.201 Results of hydrochlorothiazide standard at decreased temperature

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.105	1283865	6235	1.403	9.367
SDT2	3.108	1285452	6098	1.413	9.326
STD3	3.11	1284429	6142	1.411	9.333
STD4	3.102	1280859	6156	1.408	9.325
STD5	3.101	1284742	6041	1.412	9.266
STD6	3.104	1281294	6144	1.406	9.329
Avg	**3.105**	**1283440.17**	**6116.2**	**1.41**	**9.3158**
STDEV	0.003873	1906.25134	47.457349	0.002915	0.028012
RSD	0.1247338	0.1485267	0.7759287	0.206771	0.300699

Table 3.202 Results of amlodipine standard at decreased temperature

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	4.853	148966	8314	1.651	9.367
SDT2	4.84	151410	8332	1.647	9.326
STD3	4.841	148770	8319	1.651	9.333
STD4	4.826	149064	8328	1.642	9.325
STD5	4.828	148647	8199	1.652	9.266
STD6	4.832	150818	8304	1.648	9.329
Avg	**4.8334**	**149612.5**	**8296.4**	**1.648**	**9.3158**
STDEV	0.0068411	1187.01706	55.50045	0.003937	0.028012
RSD	0.1415371	0.79339431	0.6689703	0.238896	0.300699

Table 3.203 Results of losartan standard at decreased temperature

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	5.942	2962704	11328	1.362	5.088
SDT2	5.945	2967221	11201	1.368	5.061
STD3	5.945	2966133	11191	1.367	5.048
STD4	5.94	2950958	11229	1.363	5.112
STD5	5.94	2949927	11143	1.362	5.07
STD6	5.942	2950694	11214	1.362	5.085
Avg	**5.9424**	**2957939.5**	**11195.6**	**1.3644**	**5.0752**
STDEV	0.00251	**8263.40019**	32.677209	0.002881	0.024591
RSD	0.0422385	**0.27936339**	0.2918755	0.211153	0.484526

Table 3.204 Results of hydrochlorothiazide, a mlodipine and losartan sample at decreased temperature

	hydrochlorothiazide	amlodipine	losartan
1st trial	1283865	148966	2962704
2nd trial	1285452	151410	2967221
3rd trial	1284429	148770	2966133
Avg	1284582	149715.333	2965352.67
STDEV	804.486793	1470.8927	**2357.43766**
RSD	0.06262635	0.98245962	**0.0794994**
Recov%	**100.089**	**100.0687**	**100.2506**

iv) 5% more flow

Standard solution was injected six times while sample solution was injected three times after increasing the flow rate 5% of its optimized value. Results of hydrochlorothiazide, amlodipine and losartan standards are shown in Table 3.205, Table 3.206 and Table 3.207, respectively; results of samples for the three components are shown in Table 3.208.

Table 3.205 Results of hydrochloroth

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	1260465	5903	1.422	8.85
SDT2	3.15	1258330	5920	1.423	8.894
STD3	3.15	1260461	5953	1.422	8.927
STD4	3.148	1259495	5990	1.423	8.95
STD5	3.15	1258594	5968	1.428	8.965
STD6	3.152	1259524	5957	1.43	8.946
Avg	**3.15**	**1259478.17**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	899.135455	25.461736	0.003564	0.0273
RSD	0.0448957	0.07138952	0.4273824	0.25005	0.305494

iazide standard at increased flow rate

Table 3.206 Results of amlodipine standard at increased flow rate

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	146627	5903	1.422	8.85
SDT2	3.15	145329	5920	1.423	8.894
STD3	3.15	147866	5953	1.422	8.927
STD4	3.148	147268	5990	1.423	8.95
STD5	3.15	144190	5968	1.428	8.965
STD6	3.152	145460	5957	1.43	8.946
Avg	**3.15**	**146123.333**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	1371.82968	25.461736	0.003564	0.0273
RSD	0.0448957	0.9388163	0.4273824	0.25005	0.305494

Table 3.207 Results of losartan standard at increased flow rate

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	2898395	5903	1.422	8.85
SDT2	3.15	2899495	5920	1.423	8.894
STD3	3.15	2898858	5953	1.422	8.927
STD4	3.148	2897272	5990	1.423	8.95
STD5	3.15	2895082	5968	1.428	8.965
STD6	3.152	2896458	5957	1.43	8.946
Avg	**3.15**	**2897593.33**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	**1646.65839**	25.461736	0.003564	0.0273
RSD	0.0448957	**0.05682849**	0.4273824	0.25005	0.305494

Table 3.208 Results of hydrochlorothiazide, amlodipine and losartan sample at increased flow rate

	hydrochlorothiazide	amlodipine	losartan
1st trial	1250499	145207	2890566
2nd trial	1249927	144848	2886451
3rd trial	1249484	145729	2881331
Avg	1249970	145261.333	2886116
STDEV	508.864422	443.00602	**4626.60513**
RSD	0.04071013	0.30497174	**0.16030558**
Recov%	**99.24507**	**99.41009**	**99.6039**

v) 5% less flow

Standard solution was injected six times while sample solution was injected three times after decreasing the flow rate 5% of its optimized value. Results of hydrochlorothiazide, amlodipine and losartan standards are shown in Table 3.209, Table 3.210 and Table 3.211, respectively, results of samples for the three components are shown in table 3.212.

Table 3.209 Results of hydrochlorothiazide standard at decreased flow rate

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	1358144	5903	1.422	8.85
SDT2	3.15	1360416	5920	1.423	8.894
STD3	3.15	1359973	5953	1.422	8.927
STD4	3.148	1360208	5990	1.423	8.95
STD5	3.15	1357359	5968	1.428	8.965
STD6	3.152	1361080	5957	1.43	8.946
Avg	**3.15**	**1359530**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	1447.59566	25.461736	0.003564	0.0273
RSD	0.0448957	0.10647765	0.4273824	0.25005	0.305494

Table 3.210 Results of amlodipine standard at decreased flow rate

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	156852	5903	1.422	8.85
SDT2	3.15	156768	5920	1.423	8.894
STD3	3.15	157210	5953	1.422	8.927
STD4	3.148	156934	5990	1.423	8.95
STD5	3.15	156996	5968	1.428	8.965
STD6	3.152	157180	5957	1.43	8.946
Avg	**3.15**	**156990**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	176.635217	25.461736	0.003564	0.0273
RSD	0.0448957	0.11251367	0.4273824	0.25005	0.305494

Table 3.211 Results of losartan standard at decreased flow rate

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	3120718	5903	1.422	8.85
SDT2	3.15	3119376	5920	1.423	8.894
STD3	3.15	3119090	5953	1.422	8.927
STD4	3.148	3116600	5990	1.423	8.95
STD5	3.15	3119117	5968	1.428	8.965
STD6	3.152	3119650	5957	1.43	8.946
Avg	**3.15**	**3119091.83**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	**1359.89623**	25.461736	0.003564	0.0273
RSD	0.0448957	**0.04359911**	0.4273824	0.25005	0.305494

Table 3.212 Results of hydrochlorothiazide, aml odipine and losartan sample at decreased flow rate

	hydrochlorothiazide	amlodipine	losartan
1st trial	1351981	155688	3110151
2nd trial	1351702	156266	3100903
3rd trial	1351405	155877	3117689
Avg	1351696	155943.667	3109581
STDEV	288.046871	294.710593	**8407.50403**
RSD	0.02131003	0.18898529	**0.27037418**
Recov%	**99.42377**	**99.3335**	**99.69508**

vi) 5% more organic solvent

Standard solution was injected six times while sample solution was injected three times after increasing of organic solvent in mobile phase 5% more than optimized value. Results of hydrochlorothiazide, amlodipine and losartan standards are shown in Table 3.213, Table 3.214 and Table 3.215, respectively; results of samples for the three components Are shown in Table 3.216.

Table 3.213 Results of hydrochlorothiazide standard at increased organic solvent

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	948732	5903	1.422	8.85
SDT2	3.15	946545	5920	1.423	8.894
STD3	3.15	950544	5953	1.422	8.927
STD4	3.148	949335	5990	1.423	8.95
STD5	3.15	950452	5968	1.428	8.965
STD6	3.152	946955	5957	1.43	8.946
Avg	**3.15**	**948760.5**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	1705.35495	25.461736	0.003564	0.0273
RSD	0.0448957	0.17974557	0.4273824	0.25005	0.305494

Table 3.214 Results of amlodipine standard at increased organic solvent

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	194300	5903	1.422	8.85
SDT2	3.15	198291	5920	1.423	8.894
STD3	3.15	193719	5953	1.422	8.927
STD4	3.148	195303	5990	1.423	8.95
STD5	3.15	196519	5968	1.428	8.965
STD6	3.152	196523	5957	1.43	8.946
Avg	**3.15**	**195775.833**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	1677.4288	25.461736	0.003564	0.0273
RSD	0.0448957	0.85681096	0.4273824	0.25005	0.305494

Table 3.215 Results of losartan standard at increased organic solvent

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	3349575	5903	1.422	8.85
SDT2	3.15	3347924	5920	1.423	8.894
STD3	3.15	3352761	5953	1.422	8.927
STD4	3.148	3349366	5990	1.423	8.95
STD5	3.15	3349527	5968	1.428	8.965
STD6	3.152	3349139	5957	1.43	8.946
Avg	**3.15**	**3349715.33**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	**1612.09909**	25.461736	0.003564	0.0273
RSD	0.0448957	**0.04812645**	0.4273824	0.25005	0.305494

Table 3.216 Results of hydrochlorothiazide, amlodipine and losartan sample at increased organic solvent

	hydrochlorothiazide	amlodipine	losartan
1st trial	947706	195043	3321440
2nd trial	950579	198402	3323353
3rd trial	950502	195046	3322213
Avg	949595.667	196163.667	3322335.33
STDEV	1636.95215	1938.45411	**962.349382**
RSD	0.17238412	0.98818203	**0.02896605**
Recov%	**100.088**	**100.1981**	**99.18262**

vii) 5% less organic solvent

Standard solution was injected six times while sample solution was injected three times after decreasing of organic solvent in mobile phase 5% more than optimized value. Results of hydrochlorothiazide, amlodipine and losartan standards are shown in Table 3.217, Table 3.218 and Table 3.219, respectively; results of samples for the three components are shown in Table 3.220.

Table 3.217 Results of hydrochlorothiazide standard at decreased organic solvent

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	1309551	5903	1.422	8.85
SDT2	3.15	1309149	5920	1.423	8.894
STD3	3.15	1301371	5953	1.422	8.927
STD4	3.148	1301003	5990	1.423	8.95
STD5	3.15	1297671	5968	1.428	8.965
STD6	3.152	1306122	5957	1.43	8.946
Avg	**3.15**	**1304144.5**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	4850.84107	25.461736	0.003564	0.0273
RSD	0.0448957	0.3719558	0.4273824	0.25005	0.305494

Table 3.218 Results of amlodipine standard at decreased organic solvent

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	149040	5903	1.422	8.85
SDT2	3.15	148066	5920	1.423	8.894
STD3	3.15	150513	5953	1.422	8.927
STD4	3.148	150801	5990	1.423	8.95
STD5	3.15	148388	5968	1.428	8.965
STD6	3.152	150559	5957	1.43	8.946
Avg	**3.15**	**149561.167**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	1210.14899	25.461736	0.003564	0.0273
RSD	0.0448957	0.80913315	0.4273824	0.25005	0.305494

Table 3.219 Results of losartan standard at decreased organic solvent

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	3009295	5903	1.422	8.85
SDT2	3.15	3005498	5920	1.423	8.894
STD3	3.15	3000816	5953	1.422	8.927
STD4	3.148	3016305	5990	1.423	8.95
STD5	3.15	3007527	5968	1.428	8.965
STD6	3.152	3005727	5957	1.43	8.946
Avg	**3.15**	**3007528**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	**5149.24196**	25.461736	0.003564	0.0273
RSD	0.0448957	**0.17121177**	0.4273824	0.25005	0.305494

Table 3.220 Results of hydrochlorothiazide, amlodipine and losartan sample at decreased organic solvent

	hydrochlorothiazide	amlodipine	losartan
1st trial	1292331	148416	2988711
2nd trial	1295703	149064	2988690
3rd trial	1295874	148583	2958812
Avg	1294636	148687.667	2978737.67
STDEV	1998.01877	336.440683	**17256.1367**
RSD	0.15433054	0.22627343	**0.57931039**
Recov%	**99.2709**	**99.41596**	**99.04272**

viii) 3nm less

Standard solution was injected six times while sample solution was injected three times after decreasing the 3nm more than the optimized detection wavelength. Results of hydrochlorothiazide, amlodipine and losartan standards are shown in Table 3.221, Table 3.222 and Table 3.223, respectively; results of samples for the three components are shown in Table 3.224.

Table 3.221 Results of hydrochlorothiazide standard at decreased wavelength detection

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	1535238	5903	1.422	8.85
SDT2	3.15	1535154	5920	1.423	8.894
STD3	3.15	1542097	5953	1.422	8.927
STD4	3.148	1542123	5990	1.423	8.95
STD5	3.15	1542144	5968	1.428	8.965
STD6	3.152	1540348	5957	1.43	8.946
Avg	**3.15**	**1539517.33**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	3417.16067	25.461736	0.003564	0.0273
RSD	0.0448957	0.22196312	0.4273824	0.25005	0.305494

Table 3.222 Results of amlodipine standard at decreased wavelength detection

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	125838	5903	1.422	8.85
SDT2	3.15	124082	5920	1.423	8.894
STD3	3.15	124673	5953	1.422	8.927
STD4	3.148	123480	5990	1.423	8.95
STD5	3.15	124312	5968	1.428	8.965
STD6	3.152	123220	5957	1.43	8.946
Avg	**3.15**	**124267.5**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	936.45112	25.461736	0.003564	0.0273
RSD	0.0448957	0.75357686	0.4273824	0.25005	0.305494

Table 3.223 Results of losartan standard at decreased wavelength detection

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	3046052	5903	1.422	8.85
SDT2	3.15	3023522	5920	1.423	8.894
STD3	3.15	3029125	5953	1.422	8.927
STD4	3.148	3015176	5990	1.423	8.95
STD5	3.15	3030753	5968	1.428	8.965
STD6	3.152	3013576	5957	1.43	8.946
Avg	**3.15**	**3026367.33**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	**11927.1537**	25.461736	0.003564	0.0273
RSD	0.0448957	**0.39410793**	0.4273824	0.25005	0.305494

Table 3.224 Results of hydrochlorothiazide, amlodipine and losartan sample at decreased wavelength detection

	hydrochlorothiazide	amlodipine	losartan
1st trial	1541140	124342	3030253
2nd trial	1546986	124644	3032295
3rd trial	1537635	123647	3025713
Avg	1541920.33	124211	3029420.33
STDEV	4724.08619	511.246516	3369.07722
RSD	0.3063768	0.4115952	0.11121194
Recov%	**100.1561**	**99.95453**	**100.1009**

ix) 3nm more

Standard solution was injected six times while sample solution was injected three times after increasing the 3nm more than the optimized detection wavelength. Results of hydrochlorothiazide, amlodipine and losartan standards are shown in Table 3.225, Table 3.226 and Table 3.227, respectively; results of samples for the three components are shown in Table 3.228.

Table 3.225 Results of hydrochlorothiazide standard at increased wavelength detection

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	948589	5903	1.422	8.85
SDT2	3.15	946326	5920	1.423	8.894
STD3	3.15	946190	5953	1.422	8.927
STD4	3.148	947295	5990	1.423	8.95
STD5	3.15	948989	5968	1.428	8.965
STD6	3.152	949257	5957	1.43	8.946
Avg	**3.15**	**947774.333**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	1354.61724	25.461736	0.003564	0.0273
RSD	0.0448957	0.14292614	0.4273824	0.25005	0.305494

Table 3.226 Results of amlodipine standard at increased wavelength detection

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	194528	5903	1.422	8.85
SDT2	3.15	195558	5920	1.423	8.894
STD3	3.15	195371	5953	1.422	8.927
STD4	3.148	195671	5990	1.423	8.95
STD5	3.15	195970	5968	1.428	8.965
STD6	3.152	195843	5957	1.43	8.946
Avg	**3.15**	**195490.167**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	516.13929	25.461736	0.003564	0.0273
RSD	0.0448957	0.26402315	0.4273824	0.25005	0.305494

Table 3.227 Results of losartan standard at increased wavelength detection

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.15	3335472	5903	1.422	8.85
SDT2	3.15	3345166	5920	1.423	8.894
STD3	3.15	3344313	5953	1.422	8.927
STD4	3.148	3344193	5990	1.423	8.95
STD5	3.15	3344215	5968	1.428	8.965
STD6	3.152	3344781	5957	1.43	8.946
Avg	**3.15**	**3343023.33**	**5957.6**	**1.4252**	**8.9364**
STDEV	0.0014142	**3718.99119**	25.461736	0.003564	0.0273
RSD	0.0448957	**0.11124634**	0.4273824	0.25005	0.305494

Table 3.228 Results of hydrochlorothiazide, amlodipine and losartan sample at increased wavelength detection

	hydrochlorothiazide	amlodipine	losartan
1st trial	949786	197093	3322346
2nd trial	947980	194216	3314128
3rd trial	946508	194269	3314454
Avg	948091.333	195192.667	3316976
STDEV	1641.83353	1645.95028	**4653.41208**
RSD	0.17317251	0.84324391	**0.1402908**
Recov%	**100.0334**	**99.84782**	**99.22085**

Summary of recovery for hydrochlorothiazide, amlodipine and losartan at the nine different conditions, average and RSD are shown in Table 3.229 below.

Table 3.229 Amlodipine and atorvastatine recovery at all robustness conditions

No	Condition	hydrochlorothiazide	Amlodipine
1	Optimized conditions	99.73692799	99.63222957
2	Mor 5 degree Celsius	100.3483136	100.2771614
3	less 5 degree Celsius	100.0889666	100.0687331
4	5% More flow rate	99.24507094	99.41008737
5	5% less flow rate	99.42377145	99.3335032
6	5% more Organic solvent	100.0880271	100.1981007
7	5% less Organic solvent	99.27090135	99.41595802
8	More 3 nm	100.1560879	99.95453357
9	Less 3 nm	100.0334468	99.84781843
	Avg.	**99.74313987**	**99.76225334**

3.4.7 Assay

Standard solution and sample solution were prepared as described in section (2-4-4-11); standard solution was injected six times, while sample solution was injected three times, the average of each was used for assay calculations as shown in Table 3.230 and Table 3.231.

Table 3.230 Results of assay mixed standard

	hydrochlorothiazide	Amlodipine	Losartan
STD1	1284626	146064	2947909
SDT2	1274131	146875	2948752
STD3	1279409	147677	2948303
STD4	1277206	147013	2948774
STD5	1279045	147166	2948493
STD6	1278067	148323	2948280
Avg	**1277571.6**	**147410.8**	**2948520.4**
STDEV	2107.353743	593.3432396	236.514906
RSD	0.164949952	0.402510019	0.008021478

Table 3.231 Results assay for hydrochlorothiazide, amlodipine and losartan

	hydrochlorothiazide	Amlodipine	Losartan
1	1335350	147664	2952944
2	1311398	147664	2952944
3	1328413	147776	2948897
AVG	1325053.667	147701.3333	2953608
STDEV	12324.30186	64.66323014	2336.536539
RSD	0.930098318	0.04377972	0.079107876
%	**103.716588**	**100.1970909**	**100.1725476**

3.5 Hydrochlorothiazide, Amlodipine besylate & Valsartan

3.5.1 System Suitability

System suitability results for hydrochlorothiazide, amlodipine besylate and valsartan are shown in Table 3.232, Table 3.233 and Table 3.234, respectively.

Table 3.232 System suitability results for hydrochlorothiazide

No.	Retention time	Area	Theoretical plates	Tailing factor	Resolution
1	2.923	709252	5638	1.402	4.47
2	2.925	709372	5572	1.407	4.481
3	2.927	710620	5635	1.405	4.485
4	2.923	709524	5569	1.404	4.45
5	2.926	709821	5536	1.41	4.454
6	2.927	709669	5615	1.403	4.495
Avg	2.9256	709801.2	5585.4	1.4058	4.473
STDEV	0.00167332	487.1741988	39.44996831	0.002774887	0.019887182
RSD	**0.057195791**	**0.068635302**	**0.706305158**	**0.197388489**	**0.444605**

Table 3.233 System suitability results for Amlodipine besylate

No.	Retention time	Area	Theoretical plates	Tailing factor	Resolution
1	3.675	252945	6601	1.589	4.47
2	3.681	252710	6624	1.583	4.481
3	3.682	253058	6633	1.587	4.485
4	3.674	252722	6623	1.582	4.45
5	3.681	252573	6564	1.589	4.454
6	3.685	252141	6638	1.578	4.495
Avg	3.6806	252640.8	6616.4	1.5838	4.473
STDEV	0.004037326	331.6002111	29.9549662	0.00432435	0.019887182
RSD	**0.109692057**	**0.131253626**	**0.452738139**	**0.273036347**	**0.444605**

Table 3.234 System suitability results for valsartan

No.	Retention time	Area	Theoretical plates	Tailing factor	Resolution
1	9.25	7190341	13535	1.305	22.345
2	9.256	7187938	13611	1.305	22.376
3	9.262	7179571	13614	1.312	22.395
4	9.252	7189699	13568	1.309	22.39
5	9.258	7188237	13568	1.309	22.324
6	9.266	7176632	13594	1.31	22.38
Avg	9.2588	7184415.4	13591	1.309	22.373
STDEV	0.005403702	5894.473963	22.3383079	0.00254951	0.028425341
RSD	**0.058362881**	**0.082045283**	**0.164361032**	**0.194767743**	**0.12705199**

3.5.2 Linearity, LOD and LOQ

i) Hydrochlorothiazide

Table 3.235 shows linearity results for hydrochlorothiazide which was then treated by XLSTAT-2015 program to predict linearity data that shown in Table 3.236, Table 3.237, Figure 3.30 and Figure 3.31.

Table 3.235 linearity result for hydrochlorothiazide

Content	40	60	80	100	120	140	160
1	289178	424530	564509	710258	853951	994216	1132728
2	287727	424641	565698	711858	853505	993973	1130059
3	289244	426561	566395	708452	849832	994859	1131747
avg	**288716**	**425244**	**565534**	**710189.3**	**852429.33**	**994349.3**	**1131511.3**
STDEV	857.4231	1141.905	953.63567	1704.0379	2260.38367	457.801631	1350.01642
RSD	0.296978	0.268529	0.1686257	0.2399414	0.265169625	0.04604032	0.119310906

Figure 3.30 shows the plot of average area versus concentrations for hydrochlorothiazide in µg/ml, the linear regression equation:

$$\text{Area} = 3729.99+56478.44*\mu g/mL$$

According to ICH guidelines, acceptance criteria is $R^2 \geq 0.997$.

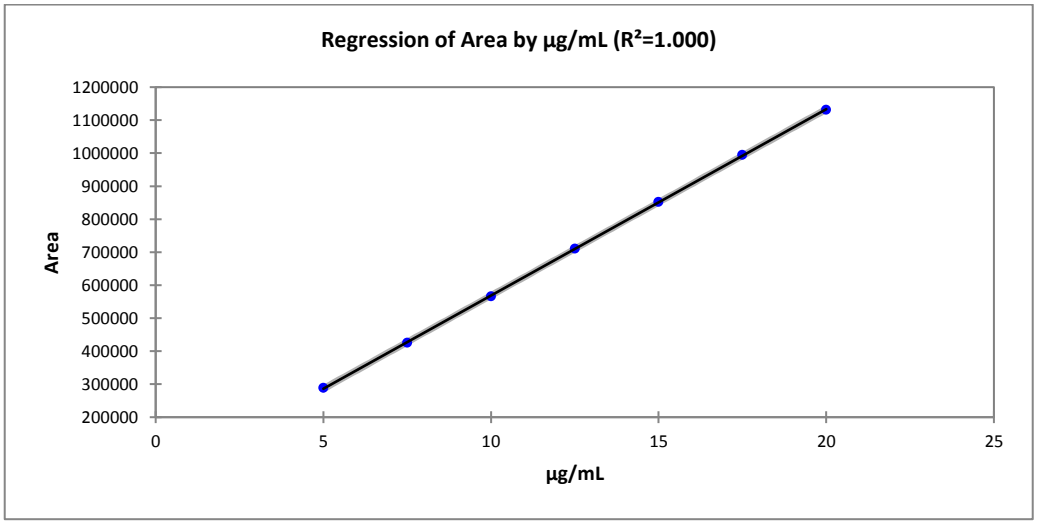

Figure 3.31 XL STAT 2015 plot of conc. in µg/ml versus average area of hydrochlorothiazide

Table 3.236 XL- STAT 2015 Goodness of fit statistics for hydrochlorothiazide

Goodness of fit statistics:	
Observations	7.000
Sum of weights	7.000
R^2	1.000
Adjusted R^2	1.000
MSE	6141085.796
RMSE	2478.121

Table 3.237 XL STAT 2015 predicted area for hydrochlorothiazide

Observation	Weight	µg/mL	Area	Pred(Area)
Obs1	1	5.000	288716.333	286122.202
Obs2	1	7.500	425244.000	427318.310
Obs3	1	10.000	565534.000	568514.417
Obs4	1	12.500	710189.333	709710.524
Obs5	1	15.000	852429.333	850906.631
Obs6	1	17.500	994349.333	992102.738
Obs7	1	20.000	1131511.333	1133298.845

Figure 3.31 is the a plot of average area versus predicted area for hydrochlorothiazide , i.e. concentration Vs predicted concentration of hydrochlorothiazide, acceptance limit for this graph is that slope ≥ 0.997

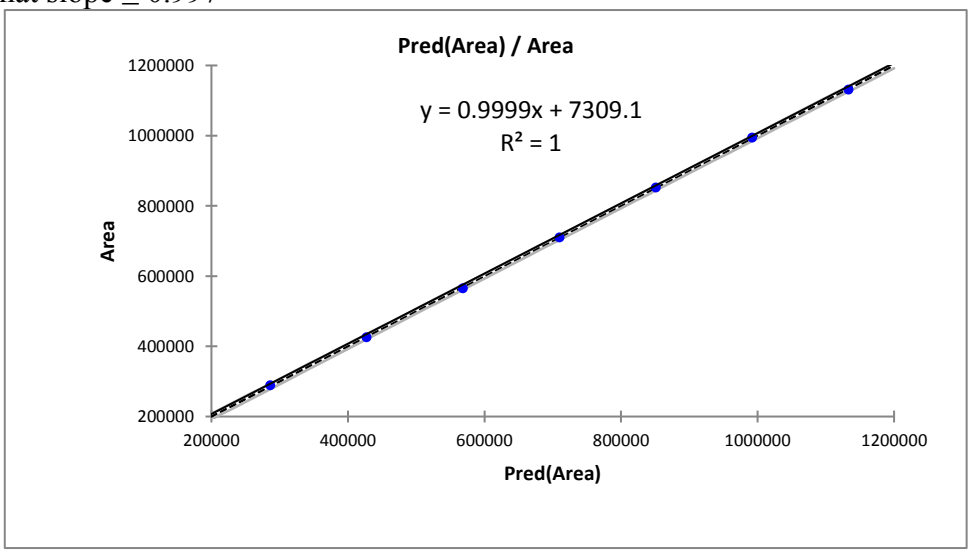

Figure 3.32 XL- STAT 2015 plot of area Vs Predicted area for hydrochlorothiazide

LOD and LOQ

LOD = 3.3* (SD/S) =3.3* (7282/50702) = **0.47µg/ml**

LOD % (relative to target concentration) = 0.47*100/12.5 = 3.7%

LOQ = 10 * (SD/S) =10* (7282/50702) = **1.44µg/ml**

LOQ % (relative to target concentration) = 1.44*100/12.5 = **11.5%**

ii) Amlodipine besylate

Table 3.238 shows linearity results for amlodipine besylate which then treated by XLSTAT-2015 program to predict linearity data that shown in Table 3.239, Table 3.240, Figure 3.32 and Figure 3.33.

Table 3.238 linearity result for amlodipine besylate

Column1	40	60	80	100	120	140	160
1	101797	151099	203305	253363	302047	352934	403446
2	101459	151208	202834	252062	301317	354620	403141
3	102018	150910	202846	251785	305441	354993	403958
avg	**101758**	**151072**	**202995**	**252403.3**	**302935**	**354182.3**	**403515**
STDEV	281.5333	150.7791	268.53491	842.55702	2200.738967	1097.05712	412.8474294
RSD	0.276669	0.099806	0.1322865	0.3338137	0.726472335	0.3097436	0.102312784

Figure 3.33 shows the plot of average area versus concentrations for amlodipine besylate in µg/ml, the linear regression equation:

$$\text{Area} = -146.99 + 14722.26 * \mu g/ml$$

According to ICH guidelines, acceptance criteria is $R^2 \geq 0.997$.

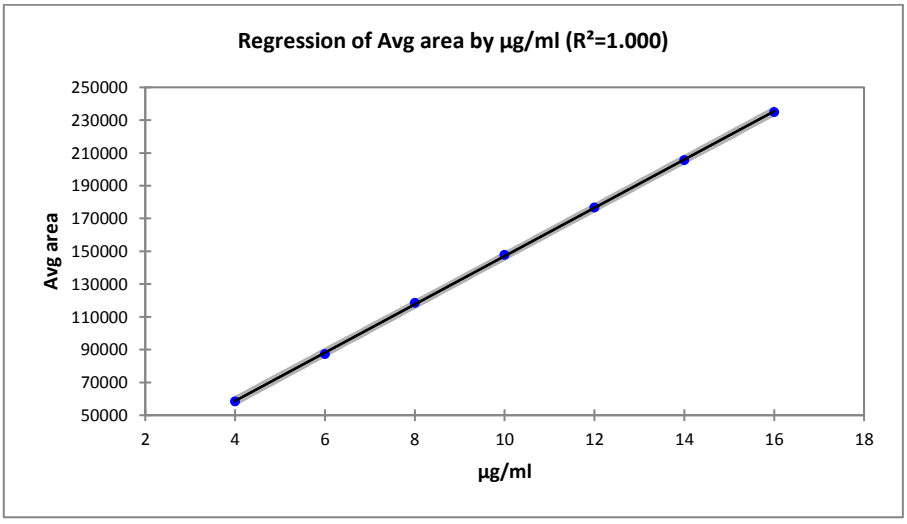

Figure 3.33 XL STAT 2015 plot of conc. in µg/ml versus average area of amlodipine

Table 3.239 XL- STAT 2015 Goodness of fit statistics for amlodipine

Goodness of fit statistics:	
Observations	7.000
Sum of weights	7.000
R^2	1.000
Adjusted R^2	1.000
MSE	526258.920
RMSE	725.437

Table 3.240 XL STAT 2015 predicted area for amlodipine besylate

Observation	Weight	µg/ml	Avg area	Pred(Avg area)
Obs1	1	4.000	58349.667	58742.036
Obs2	1	6.000	87404.333	88186.548
Obs3	1	8.000	118617.000	117631.060
Obs4	1	10.000	147772.333	147075.571
Obs5	1	12.000	176810.333	176520.083
Obs6	1	14.000	205615.333	205964.595
Obs7	1	16.000	234960.000	235409.107

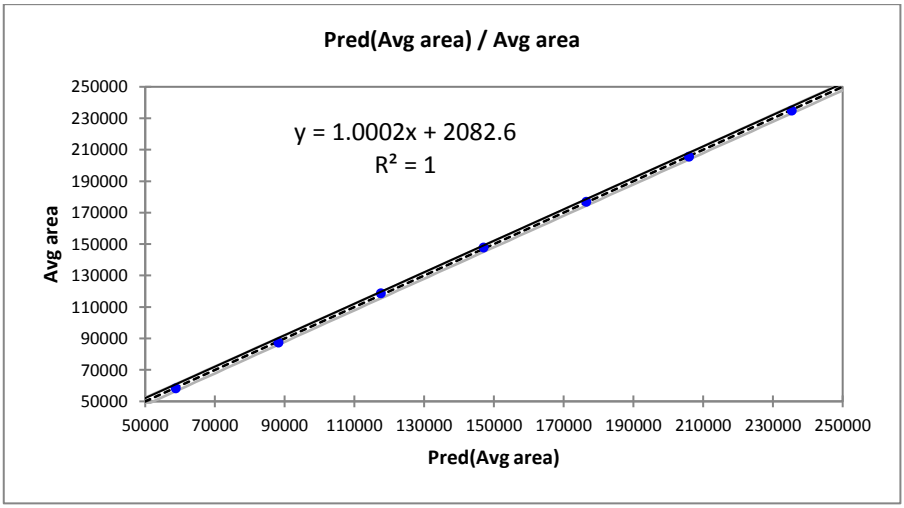

Figure 3.34 plot of average area versus predicted area for amlodipine

, i.e. concentration Vs predicted concentration of amlodipine, acceptance limit for this graph is that slope ≥ 0.997

Limit of Detection and Quantitation

LOD = 3.3* (SD/S).

LOD = 3.3* (725/14722) = **0.16 µg/ml**

LOD % (relative to target concentration) = 0.16*100/10 = 1.6%

LOQ = 10 * (SD/S). = 10* (725/14722) = **0.49 µg/ml**

LOQ % (relative to target concentration) = 0.49*100/10 = **4.9%**

iii) Valsartan

Table 3.241 shows linearity results for valsartan which then treated by XLSTAT-2015 program to predict linearity data that shown in Table 3.242, Table 3.243, Figure 3.34 and Figure 3.35.

Table 3.241 linearity result for valsartan

Column1	40	60	80	100	120	140	160
1	2899611	4314145	5763889	7199634	8656211	10107534	11494804
2	2898576	4309900	5750592	7183069	8643405	10116830	11479100
3	2901458	4283055	5761291	7197840	8632953	10100163	11549317
avg	**2899882**	**4302367**	**5758591**	**7193514**	**8644189.7**	**10108176**	**11507740**
STDEV	1459.941	16858.54	7047.7941	9090.2888	11648.8376	8352.0072	36852.65679
RSD	0.050345	0.391843	0.1223875	0.1263678	0.134759162	0.08262626	0.320242339

Figure 3.34 shows the plot of average area versus concentrations for valsartan in µg/ml, the linear regression equation:

$$\text{Area} = 1923.96 + 45000.89 * \mu g/mL$$

According to ICH guidelines, acceptance criteria is $R^2 \geq 0.997$.

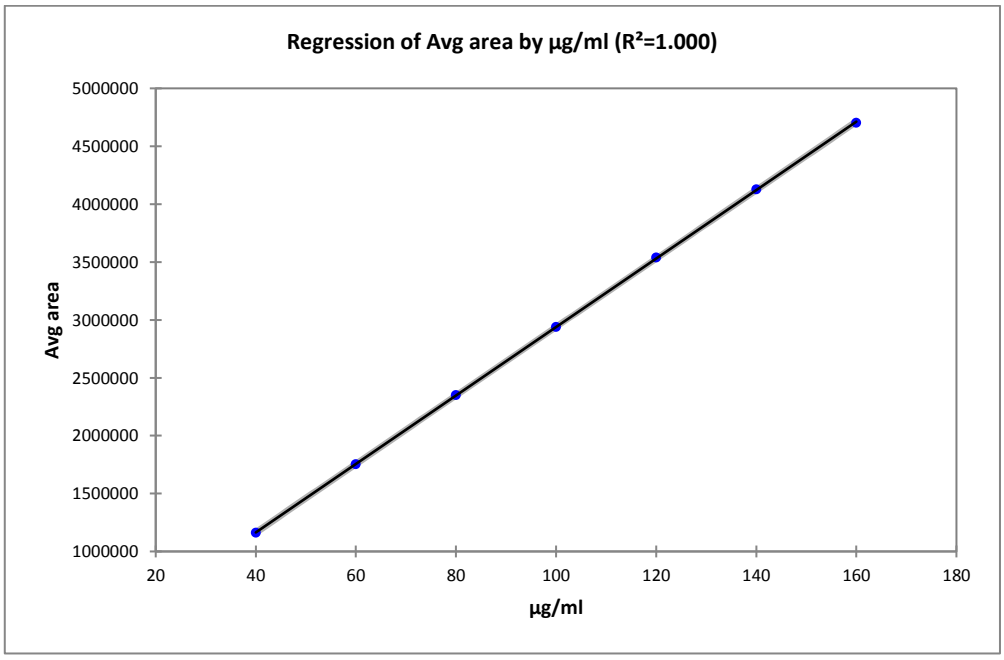

Figure 3.35 XL STAT 2015 plot of conc. in µg/ml vs average area of valsartan

Table 3.242 XL- STAT 2015 Goodness of fit statistics for valsartan

Observations	7.000
Sum of weights	7.000
R^2	1.000
Adjusted R^2	1.000
MSE	336419515.188
RMSE	18341.742

Table 3.243 XL STAT 2015 predicted area for valsartan

Observation	Weight	µg/mL	Area	Pred(Area)
Obs1	1	64.000	2899881.667	2881980.607
Obs2	1	96.000	4302366.667	4322008.929
Obs3	1	128.000	5758590.667	5762037.250
Obs4	1	160.000	7193514.333	7202065.571
Obs5	1	192.000	8644189.667	8642093.893
Obs6	1	224.000	10108175.667	10082122.214
Obs7	1	256.000	11507740.333	11522150.536

Figure 3.35 is the a plot of average area versus predicted area for valsartan, i.e. concentration Vs predicted concentration of valsartan, acceptance limit for this graph is that slope ≥ 0.997

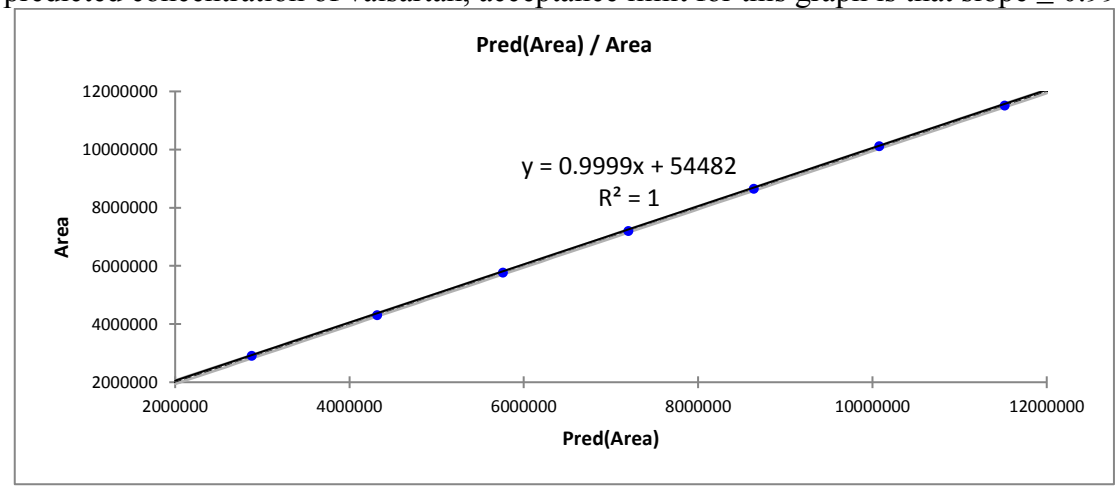

Figure 3.36 XL- STAT 2015 plot of area Vs Predicted area for valsartan

Limit of Detection and Quantitation

LOD = 3.3* (SD/S).

LOD = 3.3* (18341.7/45000.8) = **1.35 µg/ml**

LOD % (relative to target concentration) = 1.35*100/160 = 0.4%

LOQ = 10 * (SD/S).

LOQ = 10* (24414/141913) = **4.08 µg/ml**

LOD % (relative to target concentration) = 4.08*100/160 = 2.55%

3.5.3 Specificity

Figure 3.37 Figure 3.38 and Figure 3.39 shows the specificity chromatograms for placebo, sample and standard, respectively; for hydrochlorothiazide, amlodipine besylate and valsartan.

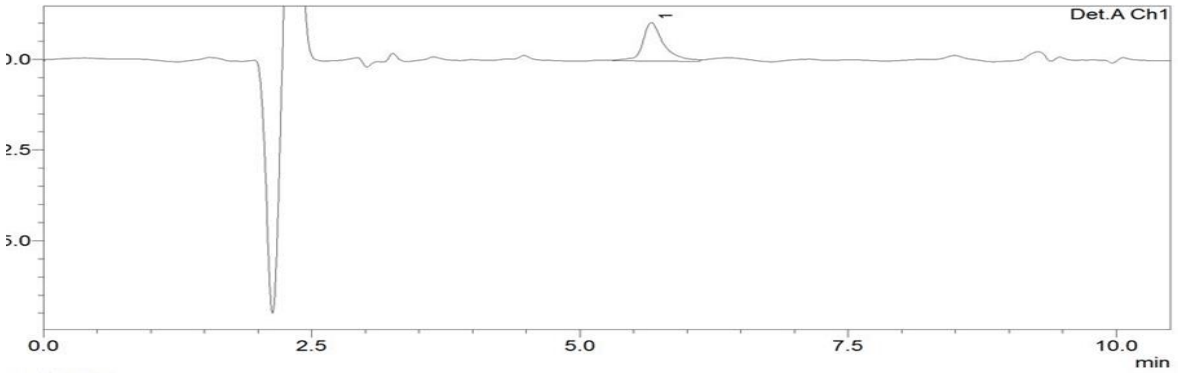

Figure 3.37 chromatogram for Placebo of hydrochlorothiazide, amlodipine besylate and valsartan

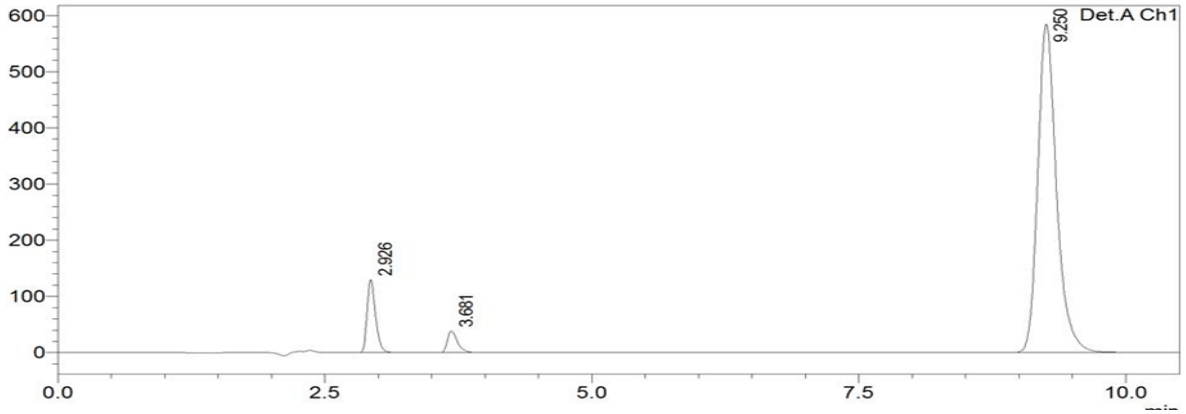

figure 3.38 chromatogram for the sample of hydrochlorothiazide, amlodipine besylate and valsartan

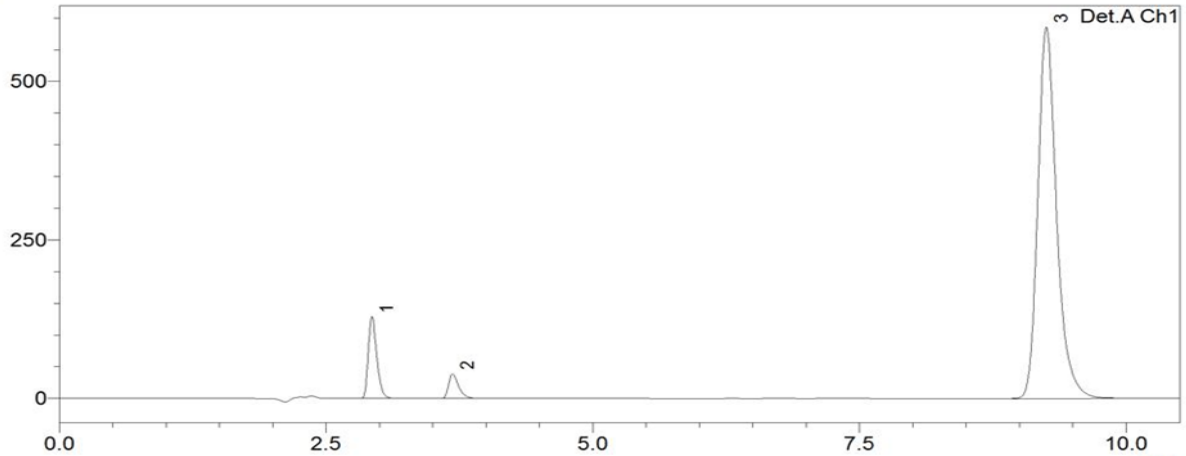

figure 3.39 chromatogram for mixed standard of hydrochlorothiazide, amlodipine and valsartan

3.5.4 Accuracy

Table 3.244 show the results of mixed standard of hydrochlorothiazide, amlodipine besylate and valsartan, while the accuracy results for samples are shown in Table 3.245, Table 3.246 and Table 3.247, respectively; summary of accuracy results for the triple mixture is shown in table 3.248.

Table 3.244 hydrochlorothiazide, amlodipine and valsartan standard for accuracy test

No.	Hydrochlorothiazide	amlodipine besylate	losartan potassium
STD1	709252	252945	7190341
SDT2	709372	252710	7187938
STD3	710620	253058	7179571
STD4	709524	252722	7189699
STD5	709821	252573	7188237
STD6	709669	252141	7176632
Avg	**709801.2**	**252640.8**	**7184415**
STDEV	**487.174199**	**331.6002**	**5894.474**
RSD	**0.0686353**	**0.131254**	**0.082045**

Table 3.245 Accuracy results for hydrochlorothiazide

Content	40	60	80	100	120	140	160
1	284672	427397	566126	711278	854337	996101	1127944
2	282365	427400	565365	711046	850319	995590	1134704
3	286439	426919	568541	711789	853591	994049	1132281
avg	**284492**	**427238.67**	**566677**	**711371**	**852749**	**995247**	**1131643**
STDEV	**2042.956**	**276.84352**	**1658.2**	**380.13**	**2137.24**	**1068.2**	**3424.862**
RSD	**0.718107**	**0.0647983**	**0.2926**	**0.0534**	**0.25063**	**0.1073**	**0.302645**
RECOVERY	**40.08052**	**60.191314**	**79.834**	**100.22**	**120.136**	**140.21**	**159.4265**
RECOVERY %	**100.2013**	**100.31886**	**99.792**	**100.22**	**100.113**	**100.15**	**99.64158**

Table 3.246 Accuracy results for amlodipine

Content	40	60	80	100	120	140	160
1	103189	150072	201021	252237	301744	355173	403465
2	101176	152874	203555	253729	303536	354221	403600
3	102601	150054	201759	251165	303423	351429	403799
avg	102322	151000	202112	252377	302901	353608	403621.3
STDEV	1035.096	1622.9566	1303.3	1287.7	1003.58	1945.9	168.0188
RSD	1.011606	1.0748057	0.6448	0.5102	0.33132	0.5503	0.041628
RECOVERY	**40.50098**	**59.768652**	**80**	**99.896**	**119.894**	**139.96**	**159.7609**
RECOVERY %	**101.2525**	**99.61442**	**100**	**99.896**	**99.9116**	**99.975**	**99.85059**

Table 3.247 Accuracy results for valsartan

Content	40	60	80	100	120	140	160
1	2891129	4293148	5861202	7200247	8603023	10119391	11467631
2	2891304	4299484	5843505	7193760	8630099	10124406	11503094
3	2875367	4299199	5855365	7187997	8609296	10123551	11505158
avg	2885933	4297277	5853357	7194001	8614139.33	10122449	11491961
STDEV	9151.131	3578.6572	9017.705	6128.565	14172.8915	2682.873	21095.656
RSD	0.317094	0.0832773	0.15406	0.08519	0.16453056	0.026504	0.1835688
RECOVERY	40.16936	59.813872	81.47298	100.1334	119.900352	140.8945	159.9568
RECOVERY %	100.4234	99.689786	101.8412	100.1334	99.9169598	100.639	99.973

Table 3.248 Summary of accuracy results for hydrochlorothazide, amlodipine and valsartan

Content%	hydrochlorothiazide	amlodipine	losartan
40	100.2013	101.25245	100.42339
60	100.31886	99.61442	99.689786
80	99.792295	99.999518	101.84123
100	100.21836	99.895583	100.13343
120	100.11315	99.911614	99.91696
140	100.15067	99.974709	100.63896
160	99.641582	99.850592	99.973
avg	**100.06232**	**100.07127**	**100.37382**
STDEV	**0.24826**	**0.5359456**	**0.7212378**
RSD	**0.2481053**	**0.5355639**	**0.7185517**

3.5.5 Precision

i) Intraday Precision

Table 3.249 show the results of hydrochlorothiazide, amlodipine and valsartan mixed standard for intraday precision test.

Table 3.249 hydrochlorothiazide, amlodipine and valsartan standard for intraday precision

No.	Hydrochlorothiazide	Amlodipine besylate	Valsartan
STD1	709252	252945	7190341
SDT2	709372	252710	7187938
STD3	710620	253058	7179571
STD4	709524	252722	7189699
STD5	709821	252573	7188237
STD6	709669	252141	7176632
Avg	**709801.2**	**252640.8**	**7184415**
STDEV	**487.1742**	**331.6002**	**5894.474**
RSD	**0.068635**	**0.131254**	**0.082045**

For intraday precision of mixed solutions containing 80%, 100% and 120% from each component, tables numbered 3.250, 3.251 and 3.252 shows results of hydrochlorothiazide, respectively; tables numbered 3.253, 3.254 and 3.255 show the results for amlodipine, while tables numbered 3.256, 3.257 and 3.258 show the intraday precision results for valsartan. Table

3.259 show the summary of the previous nine tables, the average and RSD of each five assays of the three concentrations for each active ingredient

Table 3.250 Intraday results for 80% hydrochlorothiazide

	1st	2nd	3rd	4th	5th
1st trial	569322	570135	570826	572186	572260
2nd trial	568918	570490	569502	572700	572642
3rd trial	570406	571084	570406	572852	573817
AVG	569548.667	570569.6667	570244.7	572579.3	572906
STDEV	769.460417	479.4896592	676.5836	349.0119	811.459
RSD	0.13510003	0.084037005	0.118648	0.060954	0.14164
RECOVERY	**80.24059**	**80.3844325**	**80.339**	**80.668**	**80.714**
RECOVERY %	**100.3007**	**100.480541**	**100.42**	**100.83**	**100.89**

Table 3.251 Intraday results for 100% hydrochlorothiazide

	1st	2nd	3rd	4th	5th
1st trial	849872	850519	851527	852291	850197
2nd trial	850459	851779	852723	852448	851863
3rd trial	853031	850310	851268	850803	851793
AVG	851120.667	850869.3333	851839.3	851847.3	851284
STDEV	1680.22985	794.6951198	776.1574	907.8195	942.309
RSD	0.19741382	0.093398021	0.091115	0.106571	0.11069
RECOVERY	**119.9097**	**119.874316**	**120.01**	**120.01**	**119.93**
RECOVERY %	**99.92477**	**99.8952633**	**100.01**	**100.01**	**99.944**

Table 3.252 Intraday results for 120% hydrochlorothiazide

	1st	2nd	3rd	4th	5th
1st trial	849872	850519	851527	852291	850197
2nd trial	850459	851779	852723	852448	851863
3rd trial	853031	850310	851268	850803	851793
AVG	851120.667	850869.3333	851839.3	851847.3	851284
STDEV	1680.22985	794.6951198	776.1574	907.8195	942.309
RSD	0.19741382	0.093398021	0.091115	0.106571	0.11069
RECOVERY	**119.9097**	**119.874316**	**120.01**	**120.01**	**119.93**
RECOVERY %	**99.92477**	**99.8952633**	**100.01**	**100.01**	**99.944**

Table 3.253 Intraday results for 80% amlodipine besylate

	1st	2nd	3rd	4th	5th
1st trial	742235	743788	743653	742790	743965
2nd trial	739868	744172	743167	741942	741866
3rd trial	737300	741184	739109	742220	744231
AVG	739801	743048	741976.3	742317.3	743354
STDEV	2468.182	1625.649	2495.045	432.2977	1295.491
RSD	0.333628	0.218781	0.33627	0.058236	0.1742765
RECOVERY	119.5283	120.0529	119.8797	119.9348	120.1023
RECOVERY %	99.60688	100.0441	99.89976	99.94568	100.08525

Table 3.254 Intraday results for 100% amlodipine besylate

	1st	2nd	3rd	4th	5th
1st trial	251763	251741	251030	253400	251660
2nd trial	251570	253689	251838	252765	251118
3rd trial	252659	254245	251424	253103	251622
AVG	251997.333	253225	251430.7	253089.3	251467
STDEV	581.08892	1314.90532	404.0413	317.7205	302.551
RSD	0.23059328	0.519263627	0.160697	0.125537	0.12031
RECOVERY	**99.7453**	**100.231237**	**99.521**	**100.18**	**99.535**
RECOVERY %	**99.7453**	**100.231237**	**99.521**	**100.18**	**99.535**

Table 3.255 Intraday results for 120% amlodipine besylate

	1st	2nd	3rd	4th	5th
1st trial	301817	303337	302262	301992	301104
2nd trial	303335	302944	304247	301861	303373
3rd trial	302710	304055	305757	301634	304861
AVG	302620.667	303445.3333	304088.7	301829	303113
STDEV	762.932719	563.3669615	1752.871	181.1325	1891.98
RSD	0.2521086	0.185656822	0.576434	0.060012	0.62418
RECOVERY	**119.783**	**120.109394**	**120.36**	**119.47**	**119.98**
RECOVERY %	**99.81915**	**100.091161**	**100.3**	**99.558**	**99.981**

Table 3.256 Intraday results for 80% valsartan

	1st	2nd	3rd	4th	5th
1st trial	5781441	5786208	5783584	5817201	5816053
2nd trial	5772395	5804143	5792778	5793815	5817590
3rd trial	5807905	5802741	5787775	5784733	5818844
AVG	5787247	5797697.333	5788046	5798583	5817496
STDEV	18453.245	9974.717356	4602.972	16750.91	1397.89
RSD	0.31886051	0.17204619	0.079526	0.288879	0.02403
RECOVERY	**80.55279**	**80.6982522**	**80.564**	**80.711**	**80.974**
RECOVERY %	**100.691**	**100.872815**	**100.7**	**100.89**	**101.22**

Table 3.257 Intraday results for 100% valsartan

	1st	2nd	3rd	4th	5th
1st trial	7199749	7205941	7180183	7182661	7195722
2nd trial	7198253	7204665	7188880	7193407	7200911
3rd trial	7200927	7206460	7206468	7188218	7218413
AVG	7199643	7205688.667	7191844	7188095	7205015
STDEV	1340.14775	923.7209146	13390.77	5374.05	11889.3
RSD	0.01861409	0.012819329	0.186194	0.074763	0.16501
RECOVERY	**100.212**	**100.296109**	**100.1**	**100.05**	**100.29**
RECOVERY %	**100.212**	**100.296109**	**100.1**	**100.05**	**100.29**

Table 3.258 Intraday results for 120% valsartan

	1st	2nd	3rd	4th	5th
1st trial	8606460	8605157	8607044	8617265	8616687
2nd trial	8599964	8607061	8606417	8604424	8617105
3rd trial	8605156	8605830	8608830	8609246	8612073
AVG	8603860	8606016	8607430	8610312	8615288
STDEV	3436.45399	965.5314599	1252.031	6486.49	2792.39
RSD	0.03994084	0.011219262	0.014546	0.075334	0.03241
RECOVERY	**119.7573**	**119.78729**	**119.81**	**119.85**	**119.92**
RECOVERY %	**99.79773**	**99.8227413**	**99.839**	**99.873**	**99.93**

Table 3.259 Summery of intraday results for hydrochlorothiazide, amlodipine and valsartan

	hydrochlorothiazide			amlodipine			valsartan		
	80 %	100 %	120 %	80 %	100 %	120 %	80 %	100 %	120 %
1st trial	100.30	100.18	99.92	100.23	99.75	99.82	100.69	100.21	99.80
2nd trial	100.48	100.54	99.90	100.82	100.23	100.09	100.87	100.30	99.82
3rd trial	100.42	100.11	100.01	100.36	99.52	100.30	100.70	100.10	99.84
4th trial	100.83	100.20	100.01	101.16	100.18	99.56	100.89	100.05	99.87
5th trial	100.89	100.31	99.94	101.44	99.54	99.98	101.22	100.29	99.93
Avg	**100.59**	**100.27**	**99.96**	**100.80**	**99.84**	**99.95**	**100.87**	**100.19**	**99.85**
STDEV	**0.2619**	**0.1653**	**0.0514**	**0.5164**	**0.3430**	**0.2813**	**0.2122**	**0.1094**	**0.0514**
RSD	**0.2604**	**0.1649**	**0.0514**	**0.5123**	**0.3435**	**0.2815**	**0.2104**	**0.1092**	**0.0514**

ii) Interday Precision

Table 3.260 show the results of hydrochlorothiazide, amlodipine and valsartan mixed standard for interday precision test. Tables numbered 3.261, 3.262 and 3.263 show intraday precision for 80%, 100% and 120% for the three components.Table 3.264 show the summary of interday precision, the average and RSD for each three assays of the three concentrations for each active ingredient.

Table 3.260 hydrochlorothiazide, amlodipine and valsartan standard for interday precision

	Hhydrochlorothiazide			Amlodipine			Valsartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
STD1	709252	716490	714669	709252	252564	251047	709252	7225303	7228339
SDT2	709372	714434	716160	709372	251671	251810	709372	7227314	7222419
STD3	710620	715267	718207	710620	250973	251948	710620	7230614	7227673
STD4	709524	716746	714781	709524	252740	250539	709524	7229218	7228223
STD5	709821	716877	716305	709821	253003	251851	709821	7231567	7227748
STD6	709669	716464	717063	709669	251584	251614	709669	7229197	7228857
Avg.	**709801.2**	**715957.6**	**716503.2**	**709801.2**	**251994.2**	**251552.4**	**709801.2**	**7229582**	**7226984**
STDEV	**487.174199**	**1063.202**	**1258.904**	**487.17**	**849.9216**	**579.39**	**487.174199**	**1614.76**	**2595.231**
RSD	**0.0686353**	**0.148501**	**0.175701**	**0.0686**	**0.337278**	**0.2303**	**0.0686353**	**0.022335**	**0.03591**

Table 3.261 interday precision results for 80% hydrochlorothiazide, amlodipine and valsartan

	hydrochlorothiazide			amlodipine			valsartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
assay 1	569322	575844	570215	569322	204277	203446	569322	5798396	5794262
assay 2	568918	570866	571014	568918	202038	203108	568918	5797166	5801682
assay 3	570406	571448	570916	570406	204900	204069	570406	5798546	5809176
Avg	569548.7	572719.3	570715	569548.7	203738.3	203541	569548.667	5798036	5801707
STDEV	769.5	2721.642	435.7763	769.46	1505.119	487.49	769.460417	757.1658	7457.031
RSD	0.1351	0.475214	0.076356	0.1351	0.7388	0.2395	0.13510003	0.013059	0.128532
Recovery	**80.24**	**79.99**	**79.65**	**80.24**	**80.85**	**80.91**	**80.2405894**	**80.19877**	**80.27839**
Recovery%	**100.30**	**99.99**	**99.57**	**100.30**	**101.06**	**101.14**	**100.300737**	**100.2485**	**100.348**

Table 3.262 interday precision results for 100% hydrochlorothiazide, amlodipine and valsartan

	hydrochlorothiazide			amlodipine			valsartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
assay 1	712583	716727	713570	710785	251964	254804	710785	7223752	7206692
assay 2	710785	715201	716625	709917	252647	253099	709917	7219123	7193854
assay 3	709917	716625	715167	711095	252023.3	253526.3	711095	7221003	7188934
Avg	711095	716184.3	715120.7	1359.76	596.22	1126.5	1359.76616	2433.779	20662.1
STDEV	1359.77	853.12	1528.03	0.1912	0.2366	0.4443	0.19122145	0.033704	0.287415
RSD	0.1912	0.1191	0.2137	**100.18**	**100.01**	**100.78**	**100.182276**	**99.88133**	**99.4735**
Recovery	**100.18**	**100.03**	**99.81**	**100.18**	**100.01**	**100.78**	**100.182276**	**99.88133**	**99.4735**
Recovery%	**100.18**	**100.03**	**99.81**	**100.18**	**100.01**	**100.78**	**100.182276**	**99.88133**	**99.4735**

Table 3.263 interday precision results for 120% hydrochlorothiazide, amlodipine and valsartan

	hydrochlorothiazide			amlodipine			valsartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
assay 1	849872	853078	851924	849872	302832	302261	849872	8622785	8630950
assay 2	850459	852847	850377	850459	302540	301994	850459	8622045	8611914
assay 3	853031	852869	852175	853031	305330	303455	853031	8624173	8636583
Avg	851120.70	852931.3	851492	851120.667	303567.3	302570	851120.667	8623001	8626482
STDEV	1680.23	127.4925	973.7397	1680.22985	1533.48	777.9724	1680.22985	1080.318	12927.1
RSD	0.1974	0.014948	0.114357	0.19741382	0.505153	0.257121	0.19741382	0.012528	0.149854
Recovery	**119.91**	**119.13**	**118.84**	**119.909725**	**120.466**	**120.2811**	**119.909725**	**119.2739**	**119.3649**
Recovery%	**99.92**	**99.28**	**99.033**	**99.9247708**	**100.3883**	**100.2343**	**99.9247708**	**99.39488**	**99.47075**

Table 3.264 interday precision summery for hydrochlorothiazide, amlodipine and valsartan

	hydrochlorothiazide			amlodipine			valsartan		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Day 1	80%	100%	120%	80%	100%	120%	80%	100%	120%
Day 2	100.30	100.18	99.92	100.30	100.18	99.92	100.30	100.18	99.92
Day 3	99.99	100.03	99.28	101.06	100.01	100.39	99.88	99.88	99.39
Avg	99.57	99.81	99.03	101.14	100.78	100.23	100.35	99.47	99.47
STDEV	**99.95**	**100.01**	**99.41**	**100.84**	**100.33**	**100.18**	**100.18**	**99.85**	**99.60**
RSD	**0.3689**	**0.1888**	**0.4609**	**0.4647**	**0.4062**	**0.2361**	**0.2569**	**0.3557**	**0.2866**

3.5.6 Robustness

The method was examined for robustness test under nine different conditions comparing the method output under each conditions with that of the optimized conditions and with permissible limits according to ICH, lastly the variation in method output was evaluated through calculation of the average and RSD% of the nine results obtained under the different nine conditions, detailed results were shown in the followings.

i) Optimized conditions

Standard solution was injected six times while sample solution was injected three times under optimized conditions. Results hydrochlorothiazide, amlodipine and valsartan standards are shown in Table 3.265, Table 3.266 and Table 3.267, respectively; results of samples for the three components are shown in Table 3.268.

Table 3.265 Results of hydrochlorothiazide standard at optimum conditions

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	2.923	709252	5638	1.402	4.47
SDT2	2.925	709372	5572	1.407	4.481
STD3	2.927	710620	5635	1.405	4.485
STD4	2.923	709524	5569	1.404	4.45
STD5	2.926	709821	5536	1.41	4.454
STD6	2.927	709669	5615	1.403	4.495
Avg	2.9256	709801.2	5585.4	1.4058	4.473
STDEV	0.001673	487.1742	39.4499683	0.002775	0.01989
RSD	**0.057196**	**0.068635**	**0.70630516**	**0.197388**	**0.44461**

Table 3.266 Results of amlodipine standard at optimum conditions

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.675	252945	6601	1.589	4.47
SDT2	3.681	252710	6624	1.583	4.481
STD3	3.682	253058	6633	1.587	4.485
STD4	3.674	252722	6623	1.582	4.45
STD5	3.681	252573	6564	1.589	4.454
STD6	3.685	252141	6638	1.578	4.495
Avg	3.6806	252640.8	6616.4	1.5838	4.473
STDEV	0.004037	331.6002	29.9549662	0.004324	0.01989
RSD	**0.109692**	**0.131254**	**0.45273814**	**0.273036**	**0.44461**

Table 3.267 Results of valsartan standard at optimum conditions

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	9.25	7190341	13535	1.305	22.345
SDT2	9.256	7187938	13611	1.305	22.376
STD3	9.262	7179571	13614	1.312	22.395
STD4	9.252	7189699	13568	1.309	22.39
STD5	9.258	7188237	13568	1.309	22.324
STD6	9.266	7176632	13594	1.31	22.38
Avg	9.2588	7184415	13591	1.309	22.373
STDEV	0.005404	5894.474	22.3383079	0.00255	0.02843
RSD	**0.058363**	**0.082045**	**0.16436103**	**0.194768**	**0.12705**

Table 3.268 Results of hydrochlorothiazide, amlodipine and valsartan sample at optimum conditions

	Hydrochlorothiazide	Amlodipine	Valsartan
1st trial	711278	252237	7200247
2nd trial	711046	253729	7193760
3rd trial	711789	251165	7187997
Avg	**711371**	252377	**7194001**
STDEV	**380.1302**	1287.72	**6128.565**
RSD	**0.053436**	0.510237	**0.08519**
Recov%	100.2212	**99.89558**	**100.1334**

ii) 5°C more

Standard solution was injected six times while sample solution was injected three times after the column temperature was raised up five degrees celsius, Results of hydrochlorothiazide, amlodipine and valsartan standards are shown in Table 3.269, Table 3.270 and Table 3.271, respectively; results of samples for the three components are shown in Table 3.272.

Table 3.269 Results of hydrochlorothiazide standard at increased temperature

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	2.922	721251	5675	1.342	3.826
SDT2	2.924	721120	6366	1.344	3.801
STD3	2.924	722426	5542	1.341	3.8
STD4	2.924	720204	5621	1.343	3.807
STD5	2.923	720466	5652	1.34	3.811
STD6	2.924	722180	5541	1.343	3.8
Avg	**2.9238**	**721274.5**	**5744.4**	**1.3422**	**3.8038**
STDEV	0.000447	891.0609	350.887874	0.001643	0.00497
RSD	0.015296	0.12354	6.1083468	0.122423	0.13066

Table 3.270 Results of amlodipine standard at increased temperature

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.559	254859	6427	1.514	3.826
SDT2	3.562	252235	6366	1.498	3.801
STD3	3.56	255122	6408	1.5	3.8
STD4	3.558	254095	6397	1.513	3.807
STD5	3.557	252521	6419	1.51	3.811
STD6	3.563	257494	6329	1.512	3.8
Avg	**3.56**	**254387.7**	**6383.8**	**1.5066**	**3.8038**
STDEV	0.00255	1929.496	36.4650518	0.007057	0.00497
RSD	0.071615	0.758487	0.57121231	0.4684	0.13066

Table 3.271 Results of valsartan standard at increased temperature

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	8.85	7244388	12619	1.231	21.482
SDT2	8.849	7241136	12611	1.231	21.417
STD3	8.848	7241219	12565	1.231	21.425
STD4	8.841	7243238	12621	1.23	21.443
STD5	8.84	7242809	12611	1.23	21.455
STD6	8.855	7242025	12667	1.229	21.432
Avg	**8.8466**	**7242469**	**12615**	**1.2302**	**21.4344**
STDEV	**0.006189**	**1259.051**	**36.3042697**	**0.000837**	**0.01496**
RSD	**0.069956**	**0.017384**	**0.28778652**	**0.06801**	**0.06979**

Table 3.272 Results of hydrochlorothiazide, amlodipine and valsartan sample at increased Temperature

	Hydrochlorothiazide	Amlodipine	Valsartan
1st trial	718639	251826	7250170
2nd trial	721029	254613	7251025
3rd trial	719872	254681	7257843
Avg	719846.7	253706.7	**7253013**
STDEV	1195.201	1629.06	**4204.979**
RSD	0.166036	0.642104	**0.057976**
Recov%	**99.80204**	**99.7323**	**100.1456**

iii) 5°C less

Standard solution was injected six times while sample solution was injected three times after the column temperature was decreased five celsius degrees . Results of hydrochlorothiazide, amlodipine and valsartan standards are shown in Table 3.273, Table 3.274 and Table 3.275, respectively; results of samples for the three components are shown in Table 3.276.

Table 3.273 Results of hydrochlorothiazide standard at decreased temperature

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	2.921	721640	5635	1.349	3.826
SDT2	2.922	722184	5664	1.346	3.819
STD3	2.92	720909	5589	1.353	3.804
STD4	2.921	718700	5618	1.348	3.804
STD5	2.922	720921	5639	1.345	3.815
STD6	2.922	718998	5686	1.342	3.817
Avg	**2.9214**	**720558.7**	**5639.2**	**1.3468**	**3.8118**
STDEV	0.000894	1411.051	37.9960524	0.004087	0.00726
RSD	0.030616	0.195827	0.67378445	0.303428	0.19045

Table 3.274 Results of amlodipine standard at decreased temperature

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.56	257831	6386	1.519	3.826
SDT2	3.561	259958	6302	1.521	3.819
STD3	3.557	256243	6352	1.522	3.804
STD4	3.557	254192	6374	1.519	3.804
STD5	3.558	254115	6411	1.515	3.815
STD6	3.556	253430	6423	1.516	3.817
Avg	**3.5578**	**255961.5**	**6372.4**	**1.5186**	**3.8118**
STDEV	0.001924	2549.332	48.5417346	0.00305	0.00726
RSD	0.054065	0.995983	0.76174965	0.200816	0.19045

Table 3.275 Results of valsartan standard at decreased temperature

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	8.849	7242107	12648	1.234	21.462
SDT2	8.844	7240956	12645	1.234	21.385
STD3	8.838	7237732	12679	1.234	21.449
STD4	8.836	7240380	12667	1.233	21.451
STD5	8.834	7240206	12643	1.233	21.446
STD6	8.831	7241392	12693	1.232	21.484
Avg	**8.8366**	**7240462**	**12665.4**	**1.2332**	**21.443**
STDEV	0.004879	**1505.996**	21.6055548	0.000837	0.0359
RSD	0.055208	**0.0208**	0.17058723	0.067845	0.1674

Table 3.276 Results of hydrochlorothiazide, assay mlodipine and valsartan sample at decreased temperature

	Hydrochlorothiazide	Amlodipine	Valsartan
1st trial	719023	255826	7240026
2nd trial	720018	252052	7245740
3rd trial	720813	255127	7247856
Avg	719951.3	254335	7244541
STDEV	896.8603	2007.789	**4050.435**
RSD	0.124572	0.789427	**0.05591**
Recov%	**99.91571**	**99.36455**	**100.0563**

iv) 5% more flow

Standard solution was injected six times while sample solution was injected three times after increasing the flow rate 5% of its optimized value. Results of hydrochlorothiazide, amlodipine and valsartan standards are shown in Table 3.277, Table 3.278 and Table 3.279, respectively; results of samples for the three components are shown in Table 3.280.

Table 3.277 Results of hydrochlorothiazide standard at increased flow rate

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	2.768	696305	5470	1.395	3.664
SDT2	2.764	695305	5429	1.394	3.767
STD3	2.763	694391	5498	1.391	3.778
STD4	2.765	697226	5388	1.398	3.752
STD5	2.766	696633	5408	1.395	3.751
STD6	2.767	696449	5349	1.394	3.738
Avg	**2.765**	**696051.5**	**5414.4**	**1.3944**	**3.7572**
STDEV	0.001581	1025.123	55.2657217	0.00251	0.01551
RSD	0.057184	0.147277	1.02071738	0.180004	0.41293

Table 3.278 Results of amlodipine standard at increased flow rate

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.349	239235	6404	1.55	3.664
SDT2	3.369	241801	6217	1.559	3.767
STD3	3.368	241759	6201	1.391	3.778
STD4	3.369	241030	6193	1.557	3.752
STD5	3.369	242666	6188	1.562	3.751
STD6	3.371	240002	6173	1.549	3.738
Avg	**3.3692**	**241082.2**	**6194.4**	**1.5236**	**3.7572**
STDEV	0.001095	1270.028	16.2419211	0.074282	0.01551
RSD	0.032514	0.526803	0.2622033	4.87542	0.41293

Table 3.279 Results of valsartan standard at increased flow rate

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	8.271	6960107	12833	1.283	21.429
SDT2	8.297	6947219	12973	1.283	21.324
STD3	8.292	6950738	12964	1.283	21.301
STD4	8.291	6950657	12955	1.284	21.282
STD5	8.293	6952508	12902	1.284	21.256
STD6	8.298	6951258	12845	1.286	21.217
Avg	**8.2942**	**6952081**	**12927.8**	**1.284**	**21.276**
STDEV	0.003114	**4306.897**	53.8859908	0.001225	0.04137
RSD	0.03755	**0.061951**	0.41682259	0.095385	0.19445

Table 3.280 Results of hydrochlorothiazide, amlodipine and valsartan sample at increased flow rate

	Hydrochlorothiazide	Amlodipine	Valsartan
1st trial	695835	244149	6970639
2nd trial	695623	242834	6970697
3rd trial	696818	241506	6969685
Avg	696092	242829.7	6970340
STDEV	637.6072	1321.505	**568.2758**
RSD	0.091598	0.544211	**0.008153**
Recov%	**100.0058**	**100.7249**	**100.2626**

v) 5% less flow

Standard solution was injected six times while sample solution was injected three times after decreasing the flow rate 5% of its optimized value. Results of hydrochlorothiazide, amlodipine and valsartan standards are shown in Table 3.281, Table 3.282 and Table 3.283, respectively; results of samples for yhe three components are shown in Table 3.284.

Table 3.281 Results of hydrochlorothiazide standard at decreased flow rate

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.72	269766	6561	1.55	3.811
SDT2	3.722	269768	6542	1.552	3.808
STD3	3.72	269781	6557	1.555	3.821
STD4	3.179	266305	6571	1.543	3.815
STD5	3.72	267791	6569	1.549	3.827
STD6	3.719	266533	6581	1.55	3.829
Avg	**3.612**	**268324**	**6564**	**1.5498**	**3.82**
STDEV	0.242057	1664.661	14.9666295	0.004438	0.00866
RSD	6.701462	0.620392	0.22801081	0.28639	0.22671

Table 3.282 Results of amlodipine standard at decreased flow rate

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.064	766919	5833	1.378	3.811
SDT2	3.065	765178	5791	1.376	3.808
STD3	3.062	768851	5837	1.382	3.821
STD4	3.062	769896	5805	1.386	3.815
STD5	3.06	770260	5786	1.379	3.827
STD6	3.06	767692	5784	1.377	3.829
Avg	**3.0618**	**768132.7**	**5800.6**	**1.38**	**3.82**
STDEV	0.002049	1926.039	21.9385505	0.004062	0.00866
RSD	0.066934	0.250743	0.37821175	0.294349	0.22671

Table 3.283 Results of valsartan standard at decreased flow rate

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	9.205	7682907	13277	1.269	21.798
SDT2	9.195	7672954	13225	1.269	21.723
STD3	9.18	7674472	13224	1.273	21.709
STD4	9.176	7674187	13129	1.274	21.661
STD5	9.172	7681544	13137	1.275	21.651
STD6	9.168	7682474	13148	1.275	21.657
Avg	**9.1782**	**7678090**	**13172.6**	**1.2732**	**21.6802**
STDEV	0.010402	**4670.222**	47.8570789	0.00249	0.03324
RSD	0.113333	**0.060825**	0.36330777	0.195569	0.15334

Table 3.284 Results of hydrochlorothiazide, amlodipine and losartan sample at decreased flow rate

	Hydrochlorothiazide	Amlodipine	Valsartan
1st trial	768535	266389	7703253
2nd trial	768332	269016	7709877
3rd trial	769929	267333	7708855
Avg	768932	267579.3	7707328
STDEV	869.3728	1330.711	**3566.143**
RSD	0.113062	0.497315	**0.04627**
Recov%	**100.1041**	**99.72247**	**100.3808**

vi) 5% more organic solvent

Standard solution was injected six times while sample solution was injected three times after increasing of organic solvent in mobile phase 5% more than optimized value. Results of hydrochlorothiazide, amlodipine and valsartan standards are shown in Table 3.285, Table 3.286 and Table 3.287, respectively; results of samples for the three components are shown in Table 3.288.

Table 3.285 Results of hydrochlorothiazide standard at increased organic solvent

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	2.815	720231	5281	1.434	2.542
SDT2	2.815	720218	5319	1.434	2.548
STD3	2.816	720790	5297	1.436	2.547
STD4	2.816	720159	5328	1.438	2.558
STD5	2.815	719777	5366	1.438	2.565
STD6	2.815	720579	5289	1.443	2.548
Avg	**2.8154**	**720292.3**	**5319.8**	**1.4378**	**2.5532**
STDEV	0.000548	352.8409	30.2935637	0.003347	0.00798
RSD	0.019455	0.048986	0.5694493	0.232761	0.3126

Table 3.286 Results of amlodipine standard at increased organic solvent

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.227	250245	5810	1.573	2.542
SDT2	3.227	250530	5836	1.576	2.548
STD3	3.229	252056	5832	1.577	2.547
STD4	3.229	250421	5864	1.576	2.558
STD5	3.229	250432	5882	1.583	2.565
STD6	3.228	250443	5831	1.587	2.548
Avg	**3.2284**	**250687.8**	**5849**	**1.5798**	**2.5532**
STDEV	0.000894	676.6811	22.8910463	0.00497	0.00798
RSD	0.027705	0.26993	0.39136684	0.314591	0.3126

Table 3.287 Results of valsartan standard at increased organic solvent

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	7.514	7209063	12611	1.338	19.921
SDT2	7.511	7207236	12598	1.342	19.626
STD3	7.513	7204882	12583	1.346	19.605
STD4	7.513	7205912	12626	1.348	19.648
STD5	7.512	7209229	12662	1.352	19.674
STD6	7.513	7211724	12618	1.352	19.631
Avg	**7.5124**	**7208008**	**12617.4**	**1.348**	**19.6368**
STDEV	0.000894	**2496.942**	30.0965114	0.004243	0.02584
RSD	0.011906	**0.034641**	0.2385318	0.314736	0.13159

Table 3.288 Results of hydrochlorothiazide, amlodipine and valsartan sample at increased organic solvent

	Hydrochlorothiazide	Amlodipine	Valsartan
1st trial	721908	250158	7211140
2nd trial	720374	250151	7206602
3rd trial	721142	249705	7211216
Avg	721141.3	250004.7	7209653
STDEV	767.0002	259.5425	**2642.228**
RSD	0.106359	0.103815	**0.036648**
Recov%	**100.1179**	**99.72748**	**100.0228**

vii) 5% less organic solvent

Standard solution was injected six times while sample solution was injected three times after decreasing of organic solvent in mobile phase 5% more than optimized value. Results of hydrochlorothiazide, amlodipine and valsartan standards are shown in Table 3.289, Table

3.290 and Table 3.291, respectively; results of samples for both components are shown in Table 3.292.

Table 3.289 Results of hydrochlorothiazide standard at decreased organic solvent

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.004	743814	5849	1.352	5.03
SDT2	2.989	744955	5845	1.353	4.855
STD3	2.987	741084	5822	1.357	4.935
STD4	2.987	742368	5842	1.355	4.981
STD5	2.984	744362	5779	1.354	4.967
STD6	2.985	743818	5818	1.356	4.998
Avg	**2.9864**	**743400.2**	**5821.2**	**1.355**	**4.9472**
STDEV	0.001949	1422.566	26.4140114	0.001581	0.05649
RSD	0.065275	0.191359	0.45375544	0.116689	1.14187

Table 3.290 Results of amlodipine standard at decreased organic solvent

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.873	256722	6750	1.513	5.03
SDT2	3.819	260133	6773	1.527	4.855
STD3	3.832	258137	6786	1.526	4.935
STD4	3.839	257873	6814	1.522	4.981
STD5	3.838	259540	6751	1.53	4.967
STD6	3.841	259253	6825	1.533	4.998
Avg	**3.8338**	**258609.7**	**6789.8**	**1.5276**	**4.9472**
STDEV	0.008927	1259.311	30.1114596	0.004159	0.05649
RSD	0.232863	0.486954	0.4434808	0.272279	1.14187

Table 3.291 Results of valsartan standard at decreased organic solvent

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	10.16	7430489	13611	1.23	23.419
SDT2	9.969	7435290	13125	1.232	23.046
STD3	9.986	7435703	13087	1.233	22.997
STD4	9.992	7442817	13081	1.233	22.979
STD5	9.989	7441028	13099	1.234	22.955
STD6	9.984	7442673	13060	1.235	22.946
Avg	**9.984**	**7438000**	**13090.4**	**1.2334**	**22.9846**
STDEV	0.008916	**4964.769**	23.9541228	0.00114	0.03975
RSD	0.089306	**0.066749**	0.18299	0.092442	0.17295

Table 3.292 Results of hydrochlorothiazide, amlodipine and valsartan sample at optimum conditions

	Hydrochlorothiazide	Amlodipine	Valsartan
1st trial	740302	258565	7416662
2nd trial	741717	258179	7418290
3rd trial	741245	259512	7419240
Avg	741088	258752	7418064
STDEV	720.4464	685.8928	**1303.775**
RSD	0.097215	0.265077	**0.017576**
Recov%	**99.68897**	**100.055**	**99.73197**

viii) 3nm less

Standard solution was injected six times while sample solution was injected three times after decreasing the 3nm more than the optimized detection wavelength. Results of hydrochlorothiazide, amlodipine and valsartan standards are shown in Table 3.293, Table 3.294 and Table 3.295, respectively; results of samples for both components are shown in Table 3.296.

Table 3.293 Results of hydrochlorothiazide standard at decreased wavelength detection

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	2.915	1019750	5580	1.378	3.782
SDT2	2.913	1019754	5642	1.376	3.803
STD3	2.912	1020201	5609	1.378	3.808
STD4	2.911	1018164	5578	1.379	3.786
STD5	2.909	1020776	5556	1.389	3.795
STD6	2.909	1021000	5602	1.385	3.812
Avg	**2.9108**	**1019941**	**5597.4**	**1.3814**	**3.8008**
STDEV	0.001789	1011.483	32.5392071	0.005413	0.01043
RSD	0.061456	0.099171	0.58132717	0.391845	0.27431

Table 3.294 Results of amlodipine standard at decreased wavelength detection

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.54	315672	6420	1.552	3.83
SDT2	3.541	315066	6424	1.549	3.842
STD3	3.539	314072	6375	1.553	3.823
STD4	3.539	316455	6392	1.553	3.83
STD5	3.539	313936	6381	1.555	3.829
STD6	3.538	315685	6389	1.556	3.836
Avg	**3.5392**	**315147.7**	**6392.2**	**1.5532**	**3.832**
STDEV	0.001095	990.4178	18.9921036	0.002683	0.00725
RSD	0.030952	0.314271	0.29711373	0.172758	0.18908

Table 3.295 Results of valsartan standard at decreased wavelength detection

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	8.787	6843092	13098	1.269	21.623
SDT2	8.77	6845277	13023	1.271	21.547
STD3	8.772	6846631	12996	1.272	21.516
STD4	8.766	6845171	12994	1.273	21.479
STD5	8.76	6844578	12936	1.273	21.11
STD6	8.757	6844917	13037	1.275	21.509
Avg	**8.765**	**6844944**	**12997.2**	**1.2728**	**21.4322**
STDEV	0.006403	**1146.558**	38.7517742	0.001483	0.18173
RSD	0.073053	**0.01675**	0.29815479	0.116534	0.84793

Table 3.296 Results of hydrochlorothiazide, amlodipine and valsartan sample at decreased wavelength detection

	Hydrochlorothiazide	Amlodipine	Valsartan
1st trial	1021637	318686	6864950
2nd trial	1022894	319819	6867405
3rd trial	1022304	314751	6864866
Avg	1022278	317752	6865740
STDEV	628.8929	2659.967	**1442.255**
RSD	0.061519	0.83712	**0.021007**
Recov%	**100.2292**	**100.8264**	**100.3038**

ix) 3nm more

Standard solution was injected six times while sample solution was injected three times after increasing the 3nm more than the optimized detection wavelength. Results of hydrochlorothiazide, amlodipine and valsartan standards are shown in Table 3.297, Table 3.298 and Table 3.299, respectively; results of samples for both components are shown in Table 3.300.

Table 3.297 Results of hydrochlorothiazide standard at increased wavelength detection

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	2.906	514551	5657	1.373	3.83
SDT2	2.905	512182	5647	1.375	3.842
STD3	2.904	512372	5612	1.376	3.823
STD4	2.904	513353	5619	1.378	3.83
STD5	2.903	511286	5616	1.376	3.829
STD6	2.902	516336	5633	1.381	3.836
Avg	**2.9036**	**513346.7**	**5625.4**	**1.3772**	**3.832**
STDEV	0.00114	1840.616	14.432602	0.002387	0.00725
RSD	0.039268	0.358552	0.25656135	0.173357	0.18908

Table 3.298 Results of amlodipine standard at increased wavelength detection

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	3.546	195892	6376	1.547	3.782
SDT2	3.546	195446	6384	1.545	3.803
STD3	3.547	196904	6359	1.553	3.808
STD4	3.544	195815	6297	1.545	3.786
STD5	3.544	196015	6305	1.547	3.795
STD6	3.544	196611	6352	1.55	3.812
Avg	**3.545**	**196113.8**	**6339.4**	**1.548**	**3.8008**
STDEV	0.001414	541.446	37.125463	0.003464	0.01043
RSD	0.039893	0.276088	0.58563055	0.223779	0.27431

Table 3.299 Results of valsartan standard at increased wavelength detection

No.	Ret. Time	Area	Theoretical plates	Tailing factor	Resolution
STD1	8.74	7494648	13008	1.274	21.522
SDT2	8.733	7489380	13024	1.274	21.508
STD3	8.725	7483688	12981	1.273	21.449
STD4	8.721	7489799	12997	1.272	21.453
STD5	8.719	7493300	12989	1.271	21.443
STD6	8.712	7487601	13025	1.271	21.454
Avg	**8.722**	**7489736**	**13003.2**	**1.2722**	**21.4614**
STDEV	0.007746	**3952.141**	20.2533948	0.001304	0.02641
RSD	0.08881	**0.052767**	0.155757	0.102487	0.12304

Table 3.300 Results of hydrochlorothiazide, amlodipine and valsartan sample at increased wavelength detection

	Hydrochlorothiazide	Amlodipine	Valsartan
1st trial	507115	196685	7504708
2nd trial	509894	196286	7502010
3rd trial	507255	196883	7519954
Avg	508088	196618	7508891
STDEV	1565.608	304.0872	**9675.63**
RSD	0.308137	0.154659	**0.128856**
Recov%	**98.97561**	**100.2571**	**100.2557**

Summary of recovery for hydrochlorothiazide, amlodipine and valsartan at the nine different conditions, average and RSD are shown in Table 3.301.

Table 3.301 hydrochlorothiazide, amlodipine and valsartan recovery at all robustness conditions

No	Condition	Hydrochlorothiazide		Valsartan
1	Optimized conditions	**100.2212**	**99.895583**	**100.1334**
2	Mor 5 degree Celsius	**99.80204**	**99.7322983**	**100.1456**
3	less 5 degree Celsius	**99.91571**	**99.3645529**	**100.0563**
4	5% More flow rate	**100.0058**	**100.724857**	**100.2626**
5	5% less flow rate	**100.1041**	**99.7224748**	**100.3808**
6	5% more Organic solvent	**100.1179**	**99.7274831**	**100.0228**
	5% less Organic solvent	**99.68897**	**100.055038**	**99.73197**
	More 3 nm	**100.2292**	**100.257079**	**100.3038**
	Less 3 nm	**98.97561**	**100.826385**	**100.2557**
	Avg.	**99.8956**	**100.033972**	**100.1437**
	STDEV	**0.390593**	**0.48794297**	**0.193789**
7	**RSD %**	**0.391002**	**0.48777727**	**0.193511**

3.5.7 Assay

Standard solution and sample solution were prepared as described in section (2-4-5-11); standard solution was injected six times, while sample solution was injected three times, the average of each was used for assay calculations as shown in Table 3.302 and Table 3.303.

Table 3.302 Results of assay mixed standard

	Hydrochlorothizide	**Amlodipine**	**Valsartan**
1	721661	252729	7235929
2	722521	255778	7245100
3	722312	255836	7247793
4	721528	254118	7246361
5	720214	252470	7243527
6	721397	254503	7248566
Avg	**721594.4**	**254541**	**7246269.4**
STDEV	**911.3749503**	**1385.334617**	**2030.54015**
RSD	**0.12630017**	**0.544248124**	**0.02802187**

Table 3.303 Results assay for hydrochlorothiazide, amlodipine and losartan

	Hydrochlorothizide	**Amlodipine**	**Valsartan**
1st trial	716432	254182	7197367
2nd trial	717829	254702	7194371
3rd trial	722911	255182	7232375
AVG	719057.3333	254688.6667	7208037.67
STDEV	3409.686545	500.1333156	21129.916
RSD	0.474188411	0.196370464	0.29314381
Assay	**99.64840821**	**100.0580129**	**99.4723943**

4.1 Discussion

4.1.1 Hydrochlorothiazide and valsartan

A simple and sensitive RP-HPLC method was developed for the determination of hydrochlorothiazide and valsartan in their combined pharmaceutical formulations. The separation was achieved using Thermo - phenyl hexyl column (150 × 4.6 mm, 3 μm particle size), both components were determined by UV detector at fixed wavelength at 275nm, for simplicity of the method an isocratic elution was selected, the optimized mobile phase was composed of methanol and 1% formic acid solution at 75: 25 ratio, with flow rate of 0.8 ml/min, injection volume was 20 μl, and the separation was performed at ambient temperature. Linearity of this method was checked using seven solutions centered with the target concentration, the concentrations range was (2.5–10) μg/ml for hydrochlorothiazide and (5–20) μg/ml for valsartan. Each solution was injected in triplicate. Plot of average area versus prepared concentrations indicates a very good linearity correlation, (R^2 =1) for both components. The limit of detection for hydrochlorothiazide and valsartan was found to be 0.056 μg/ml and 0.53 μg/ml, respectively; the percentage of limit of detection for hydrochlorothiazide and valsartan was 0.9% and 1.3%, respectively; whereas the limit of quantitation was found to be 0.17 μg/ml and 1.6 μg/ml, respectively; and percentage of limit of quantitation for hydrochlorothiazide and valsartan is 2.7% and 4%, respectively. Limit of detection and limit of quantitation were within the acceptance limits since the percentage of limit of detection relative to target concentration was not more than 5% and percentage of limit of quantitation relative to target concentration was not more than 20%. In specificity tests ,non of placebo peaks had same retention time of active ingredients peaks. This indicates that the excipients used in the formulation did not interfere in the estimation when we

used this method for assay in tablets. Accuracy was evaluated for hydrochlorothiazide and valsartan using seven concentrations in content of 40%, 60%, 80%, 100%, 120%, 140% and160% of target concentration. The recovery percentage for hydrochlorothiazide at the above concentrations was found to be 100.4, 100.1, 100.03, 100.87, 100, 100.89 and 100.78, respectively; while for valsartan it was 100.53, 101.2, 100.6, 100.3, 99.85, 100.6 and 99.95, respectively. The average of recovery percentage for hydrochlorothiazide and valsartan was 100.44% and 100.21%, respectively. The precision of the methods was examined by estimating the corresponding recovery percentages five times on the same day in intraday precision and three times at three different days for inter day precision. The concentrations used was 80%,100% and 120% of target concentration as per ICH. For hydrochlorothiazide intraday precision, the RSD for the recovery percentage of five assay repetitions was 0.23%, 0.16% and 0.15% for 80%, 100% and 120%, respectively; whereas for valsartan RSD was 0.15, 0.16 and 0.19, for 80%, 100% and 120%, respectively. For the interday , the RSD for the recovery percentage of hydrochlorothiazide three assay repetitions was 0.33%, 0.41% and 0.0.16% for 80%, 100% and 120%, respectively; whereas for valsartan RSD was 0.12, 0.25 and 0.37 for 80%, 100% and 120%, respectively. The RSD values was found to be not more than 2.0% so it's acceptable according to USP and ICH. The robustness of the method was assessed by assaying test solutions under different analytical conditions deliberately changed from the original conditions such as flow rate, mobile phase composition, detection wavelength and column temperature. RSD for the recovery at all different conditions for target concentration was calculated and were found to be 0.473 for hydrochlorothiazide and 0.265% for valsartan.

4.1.2 Amlodipine besylate and losartan potassium

A simple sensitive RP-HPLC method was developed for the determination of amlodipine and losartan in their combined pharmaceutical formulations. The separation was achieved using Thermo - phenyl hexyl column (150 × 4.6 mm, 3 μm particle size), both components were determined by UV detector at fixed wavelength at 260nm, for simplicity of the method an isocratic elution was selected, the optimized mobile phase was composed of acetonitrile and 1% formic acid solution at 60: 40 ratio, with flow rate of 0.8 ml/min, injection volume was 20μl and the separation was performed at ambient temperature. Linearity was checked using seven solutions centered with the target concentration, the concentrations range was 8 μg/ml –32μg/ml for amlodipine and 80 μg/ml –320μg/ml for losartan potassium. Each solution was injected in triplicate. Plot of average area versus prepared concentrations indicates a very good linearity correlation**, (**R^2 =1) for both components **.** The limit of detection for amlodipine and losartan was found to be 0.19 μg/ml and 1.6 μg/ml, respectively; the percentage of limit of detection for amlodipine and losartan potassium was 0.95% and 0.8%, respectively; whereas the limit of quantitation was found to be 0.57μg/ml and 4.8μg/ml, respectively, and percentage of limit of quantitation for amlodipine and losartan potassium was 2.85% and 2.4% ,lrespectively. Limit of detection and limit of quantitation were within the acceptance limits since the percentage of limit of detection relative to target concentration was not more than 5% and percentage of limit of quantitation relative to target concentration is not more than 20%. In specificity tests ,non of placebo peaks had same retention time of active ingredients peaks. This indicates that the excipients used in the formulation did not interfere in the estimation when we used this method for assay in tablets. Accuracy was evaluated for amlodipine and losartan potassium using seven concentrations in content of 40%,

60%, 80%, 100%, 120%, 140% and 160% of target concentration. The recovery percentage for amlodipine at the above concentrations was found to be 99.94, 99.93, 100.33, 99.71, 99.89, 100.16 and 99.76, respectively; for losartan it was 100.3, 100.2, 100.6, 99.94, 99.25, 99.23 and 99.49, respectively. The average of recovery percentage for amlodipine and losartan potassium was 99.94% and 99.36%, respectively. The precision of the methods was examined by estimating the corresponding recovery percentages five times on the same day in intraday precision and three times at three different days for inter day precision. The concentrations used was 80%,100% and 120% of target concentration as per ICH. For amlodipine intraday precision, the RSD for the recovery percentage of five assay repetitions was 0.04%, 0.08% and 0.12%, for 80%, 100% and 120%, respectively, whereas for losartan the RSD was 0.03, 0.04 and 0.03 for 80%, 100% and 120% respectively. For the interday , the RSD for the recovery percentage ofamlodipine three assay repetitions was 0.33%, 0.14% and 0.07%, for 80%, 100% and 120%, respectively; whereas for losartan the RSD was 0.07, 0.17 and 0.17 for 80%, 100% and 120%, respectively. The RSD values was found to be not more than 2.0% so it's acceptable according to USP and ICH. The robustness of the method was assessed by assaying test solutions under different analytical conditions deliberately changed from the original conditions such as flow rate, mobile phase composition, detection wavelength and column temperature. RSD for the recovery at all different conditions for target concentration was calculated and were found to be 0.26 for amlodipine and 0.18% for losartan.

4.1.3 Amlodipine besylate and atorvastatin calcium

A simple and sensitive RP-HPLC method was developed for the determination of Amlodipine besylate and atorvastatin calcium in their combined pharmaceutical formulations. The separation was achieved using Neucleodur polaratic - (50mmx2mm I.D, 1.8 µm), both components were determined by UV detector at fixed wavelength at 240nm, simple isocratic elution was selected, the optimized mobile phase was composed of methanol and 1% formic acid solution at 60: 40 ratio, with flow rate of 0.3 ml/min, injection volume was 20 µl and the separation performed at ambient temperature (column oven is not required). Linearity of this method was checked using seven solutions centered with the target concentration, the concentrations range was 8ng–32ng for amlodipine and atorvastatin calcium. Each solution was injected in triplicate. Plot of average area versus prepared concentrations indicates a very good linearity correlation which was found to be (R^2 =0.999) and (R^2 =1.000) for Amlodipine besylate and atorvastatin calcium respectively . The limit of detection for amlodipine besylate and atorvastatin calcium was found to be 0.96ng and 0.57ng, respectively, percentage of limit of detection for Amlodipine besylate and atorvastatin calcium is 4.8% and 2.85%, respectively; whereas the limit of quantitation was found to be 2.9ng and 1.7ng, respectively. The percentage of limit of quantitation for hydrochlorothiazide and valsartan was 14.5% and 8.5%, respectively; limit of detection and limit of quantitation were within the acceptance limits since the percentage of limit of detection relative to target concentration was not more than 5% and percentage of limit of quantitation relative to target concentration was not more than 20%. In specificity tests ,non of placebo peaks had same retention time of active ingredients peaks. This indicates that the excipients used in the formulation did not interfere in the estimation when we used this method for assay in tablets. Accuracy was evaluated for Amlodipine besylate and atorvastatin calcium using

seven concentrations in content of 40%, 60%, 80%, 100%, 120%, 140% and 160%, of target concentration. The recovery percentage for Amlodipine besylate at the above concentrations was found to be 99.07, 99.52, 99.85, 100.09, 99.74, 100.18 and 100.13, respectively; while for atorvastatin calcium, it was 98.17, 98.01, 99.15, 99.40, 99.60, 99.06 and 100.49, respectively. The average of recovery percentage for Amlodipine besylate and atorvastatin calcium was 100.01% and 99.72%, respectively. The precision of the methods was examined by estimating the corresponding recovery percentages five times on the same day in intraday precision and three times at three different days for inter day precision. The concentrations used was 80%,100% and 120% of target concentration according to USP and ICH. For amlodipine besylate intraday precision, the RSD for the recovery percentage of five assay repetitions was 0.55%, 0.28% and 0.21%, for 80%, 100% and 120%, respectively; whereas for atorvastatin calcium RSD was 0.42%, 0.28% and 0.21%, for 80%, 100% and 120%, respectively. For the interday , the RSD for the recovery percentage of amlodipine three assay repetitions was 0.34%, 0.37% and 0.35%, for 80%, 100% and 120%, respectively; whereas for atorvastatin calcium RSD was 0.39, 0.13 and 0.25, for 80%, 100% and 120%, respectively. The RSD values was found to be not more than 2.0% so it's acceptable according to USP and ICH. The robustness of the method was assessed by assaying test solutions under different analytical conditions deliberately changed from the original conditions such as flow rate, mobile phase composition, detection wavelength and column temperature. RSD for the recovery at all different conditions for target concentration was calculated and were found to be 0.26 for amlodipine besylate and 0.18% for atorvastatin calcium.

4.1.4 Amlodipine besylate, hydrochlorothiazide and losartan potassium

A simple and sensitive RP-HPLC method was developed for the determination of amlodipine besylate, hydrochlorothiazide and losartan potassium in their combined pharmaceutical formulations. The separation was achieved using Phenyl hexyle column (150mm × 4.6 mm, 3 µm), the thre components were determined by UV detector, at fixed wavelength at 260nm, for simplicity of the method an isocratic elution was selected, the optimized mobile phase was composed of acetonitrile and 1% formic acid solution at 1:1 ratio, with flow rate of 0.8 mL/min, injection volume was 20 µl and the separation was performed at ambient temperature. Linearity was checked using seven solutions centered with the target concentration, the concentrations range was 4µg/ml-16µg/ml, 10µg/ml–40µg/ml and 40µg/ml -160µg/ml, for amlodipine besylate, hydrochlorothiazide and losartan potassium, respectively. Each solution was injected in triplicate. Plot of average area versus prepared concentrations indicates a very good linearity correlation (R^2) was found to be 1.000 for each of the three components. The limit of detection for amlodipine besylate, hydrochlorothiazide and losartan potassium was found to be 0.16µg/ml, 0.47µg/ml and 0.19µg/ml, respectively; percentage of limit of detection was 1.6%, 1.88 and 0.78%, respectively, whereas the limit of quantitation was found to be 0.49%, 1.44 and 2.37%, respectively; the percentage of limit of quantitation was 4.9%, 5.76 and 2.37%, respectively; limit of detection and limit of quantitation were within the acceptance limits since the percentage of limit of detection relative to target concentration was not more than 5% and percentage of limit of quantitation relative to target concentration was not more than 20%. In specificity tests ,non of placebo peaks had same retention time of active ingredients peaks. This indicates that the excipients used in the formulation did not interfere in the estimation when we use this method for assay in tablets. Accuracy was evaluated for amlodipine besylate, hydrochlorothiazide

and losartan potassium using seven concentrations in content of 40%, 60%, 80%, 100%, 120%, 140% and160%, of target concentration. The recovery percentage for Amlodipine besylate was found to be 99.08, 99.25, 100.66, 99.63, 100.32, 99.97 and 99.67, respectively; for hydrochlorothiazide it was 100.82, 100.17, 101.16, 99.74, 100.06, 99.40 and 98.76, while it was 98.03, 99.73, 102.29, 99.54, 100.61, 100.29 and 100.24, for losartan. The average of recovery percentage for Amlodipine besylate hydrochlorothiazide and losartan was 99.41%, 99.99% and100.38%, respectively. The precision of the methods was examined by estimating the corresponding recovery percentages five times on the same day, in intraday precision and three times at three different days for inter day precision. The concentrations used was 80%,100% and 120%, of target concentration according to USP and ICH. For amlodipine besylate intraday precision, the RSD for the five assay repetitions was 0.26%, 0.29% and 0.21%, for 80%, 100% and 120%, respectively; for hydrochlorothiazide RSD was 0.13, 0.17 and 0.03, for 80%, 100% and 120%, respectively; RSD for 80%, 100% and 120%, losartan was 0.06%, 0.09% and 0.13%, respectively. In interday the RSD for the recovery percentage of 80%, 100% and 120%, amlodipine three assay repetitions was 0.06%, 0.45% and 0.14%, for 80%, 100% and 120%, respectively, whereas for 80%, 100% and 120%, hydrochlorothiazide the RSD was 0.10%, 0.50% and 0..07%, respectively, and 0.06%, 0.45% and 0.14%, for 80%, 100% and 120%, losartan respectively. The RSD values were not more than 2.0% so it's acceptable according to USP and ICH. The robustness of the method was assessed by assaying test solutions under different analytical conditions deliberately changed from the original conditions such as flow rate, mobile phase composition, detection wavelength and column temperature. RSD for the recovery at all different conditions for target concentration were 0.36%, 0.41% and 0.46%, for amlodipine besylate hydrochlorothiazide, and losartan, respectively.

4.1.5 Amlodipine besylate, hydrochlorothiazide and valsartan

A simple and sensitive RP-HPLC method was developed for the determination of amlodipine besylate, hydrochlorothiazide and valsartan in their combined pharmaceutical formulations. The separation was achieved using Phenyl hexyle column (150mm × 4.6 mm, 3 µm), the thre components were determined by UV detector at fixed wavelength at 254nm, for simplicity of the method an isocratic elution was selected, the mobile phase optimized was composed of acetonitrile and 1% formic acid solution at 1:1 ratio, with flow rate of 0.8 mL/min, injection volume was 20 µl and the separation performed at ambient temperature (column oven is not required). Linearity of this method was checked using seven solutions centered with the target concentration, the concentrations range was 4µg/ml-14µg/ml , 5µg/ml–20µg/ml and 64µg/ml-256µg/ml, for amlodipine besylate, hydrochlorothiazide and valsartan respectively. Each solution was injected in triplicate. Plot of average area versus prepared concentrations indicates a very good linearity correlation (R^2) was found to be 1.000 for each of the three components. The limit of detection for amlodipine besylate, hydrochlorothiazide and valsartan was found to be 0.16, 0.47 and 1.35 µg/ml respectively, percentage of limit of detection was 1.6%, 3.7% and 0.4% respectively, the limit of quantitation was found to be 0.49%, 1.44 and 4.08% respectively and percentage of limit of quantitation was 4.9%, 11.5 and 2.55% respectively, limit of detection and limit of quantitation were within the acceptance limits since the percentage of limit of detection relative to target concentration is not more than 5% and percentage of limit of quantitation relative to target concentration is not more than 20%. In specificity tests ,non of placebo peaks had same retention time of active ingredients peaks which indicates that excipients used in the formulation do not interfere in the estimation when we use this method for assay in tablets. Accuracy was evaluated for amlodipine besylate, hydrochlorothiazide and

valsartan using seven concentrations in content of 40%, 60%, 80%, 100%, 120%, 140% and160%, of the target concentration. The recovery percentage for Amlodipine besylate was found to be 100.2, 100.3, 99.8, 100.2, 100.1, 100.2 and 99.6, respectively; for hydrochlorothiazide it was 101.3, 99.6, 100.0, 99.9, 99.9, 100.0 and 99.9, while it was 100.4, 99.7, 101.8, 100.1, 99.9, 100.6 and 100.0, for valsartan. The average of recovery percentage for Amlodipine besylate hydrochlorothiazide and valsartan was 100.06%, 100.07% and 100.37%, respectively. The precision of the methods was examined by estimating the corresponding recovery percentages five times on the same day, in intraday precision and three times at three different days for inter day precision. The concentrations used was 80%,100% and 120%, of target concentration according to the USP and ICH. For amlodipine besylate intraday precision, the RSD for the five assay repetitions was 0.51%, 0.34% and 0.28%, for 80%, 100% and 120%, respectively; for hydrochlorothiazide RSD was 0.26, 0.16 and 0.05, for 80%, 100% and 120%, respectively; RSD for 80%, 100% and 120%, valsartan was 0.21%, 0.11% and 0.05%, respectively. In interday test the RSD for the recovery percentage of 80%, 100% and 120%, amlodipine three assay repetitions was 0.46%, 0.40%, and 0.24% for 80%, 100% and 120%, respectively; whereas for 80%, 100% and 120%, hydrochlorothiazide the RSD was 0.37%, 0.19% and 0.46%, respectively; and for valsartan it was 0.26%, 0.36% and 0.29%, for 80%, 100% and 120%, respectively. The RSD values were not more than 2.0% so it's acceptable according to USP and ICH. The robustness of the method was assessed by assaying test solutions under different analytical conditions deliberately changed from the original conditions such as flow rate, mobile phase composition, detection wavelength and column temperature. RSD for the recovery at all different conditions for target concentration were 0.49%, 0.39%

and 0.19%, for amlodipine besylate hydrochlorothiazide, and valsartan respectively.

4.1.6 Final statement discussion

System suitability parameters at all different conditions were found to be within the accepted limit of USP and ICH guidelines. This indicates that these analytical methods gives results with high reality even if slight but deliberate changes occur in the analytical conditions therefore its recommended for the analysis of this drug for quality control routine work and for research purposes.

4.2.Recommenditions

Since these combinations are very important as antihypertensive drugs, and official methods are not available for, It is recommended to develop new methods for these antihypertensive drugs, using other techneaques polarography, spectrophotometry or even liquid chromatography but with different detection systems, for example, refractive index detector or fluorescence detector, it might be beneficial also to develop an isocratic HPLC methods for other multicomponent drugs.

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==== Shimadzu LCsolution Analysis Report ====

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Vail #	: 1
Injection Volume	: 5 uL
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Method File Name	: HCTZ - VALS.lcm
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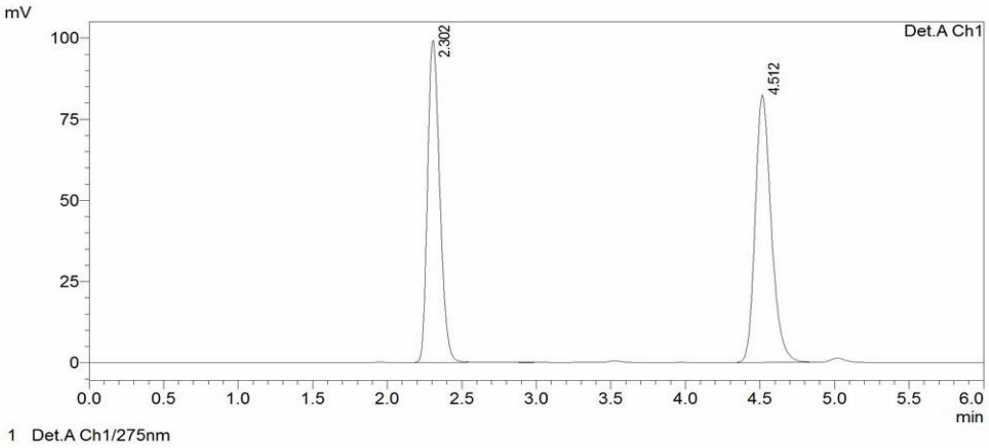

1 Det.A Ch1/275nm

PeakTable

Detector A Ch1 275nm

Ret. Time	Area	Theoretical Plate#	Tailing Factor	Resolution
2.302	562357	3219.560	1.346	0.000
4.512	619051	7712.346	1.293	12.020

C:\LabSolutions\BABFAT\HCTZ - VALS SYS SUIT. 1.lcd

Appendix I System suitability test for hydrochlorothiazide and valsartan (Injection No. 1)

==== Shimadzu LCsolution Analysis Report ====

F:\amlo los ACN\2 Linearity\Linearity 40 % -1.lcd

Acquired by	: Admin
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Sample ID	: Linearity 40 % -1
Tray#	: 1
Vail #	: 2
Injection Volume	: 20 uL
Data File Name	: Linearity 40 % -1.lcd
Method File Name	: Amlo-los(ACN).lcm
Batch File Name	: Amlo-los(ACN).lcb
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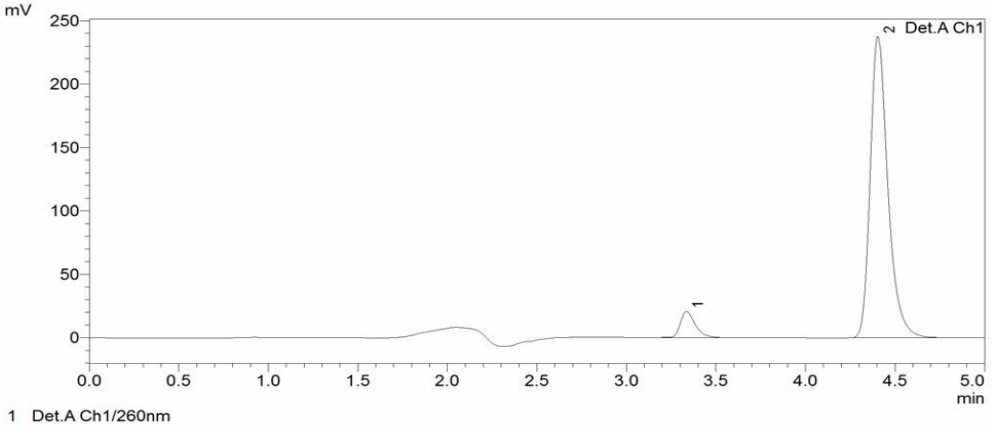

1 Det.A Ch1/260nm

PeakTable

Detector A Ch1 260nm

Ret. Time	Area	Theoretical Plate#	Tailing Factor	Resolution
3.331	123504	6558.916	1.526	0.000
4.400	1610876	9046.832	1.395	6.118

Appendix II Linearity for amlodipine and losartan (40% of target concentration, Injection No. 1)

==== Shimadzu LCsolution Analysis Report ====

C:\LabSolutions\Data\Project1\MedOne\SVB1104\STVBWB2.5H\Am-At Accuracy 40% - 1.lcd

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Sample ID	: Am-At Accuracy 40% - 1
Tray#	: 1
Vail #	: 11
Injection Volume	: 20 uL
Data File Name	: Am-At Accuracy 40% - 1.lcd
Method File Name	: Amilo-Atro.lcm
Batch File Name	: Amilo-Atro.lcb
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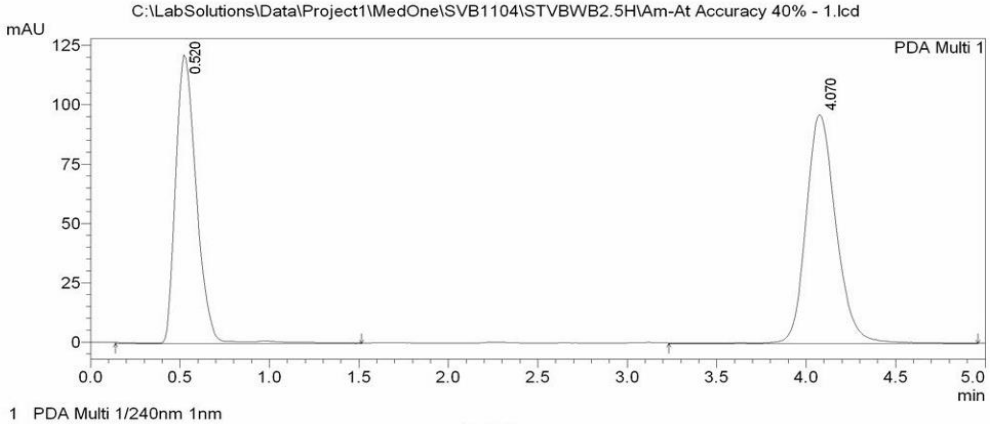

1 PDA Multi 1/240nm 1nm

PeakTable

PDA Ch1 240nm 1nm

Peak#	Ret. Time	Area	Area %	Tailing Factor	Resolution
1	0.520	995515	46.657	1.390	0.000
2	4.070	1138153	53.343	1.227	13.230
Total		2133668	100.000		

Appendix III Accuracy for amlodipine and atorvastatin (40% of target concentration, Injection No. 1)

==== Shimadzu LCsolution Analysis Report ====

F:\hctz amlo los\5 itra\AmloHctz-los Prec 1st intra 80 -1.lcd

Acquired by	: Admin
Sample Name	: AmloHctz-los Prec 1st intra 80 -1
Sample ID	: AmloHctz-los Prec 1st intra 80
Tray#	: 1
Vail #	: 10
Injection Volume	: 20 uL
Data File Name	: AmloHctz-los Prec 1st intra 80 -1.lcd
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Batch File Name	: AmloHctz-los.lcb
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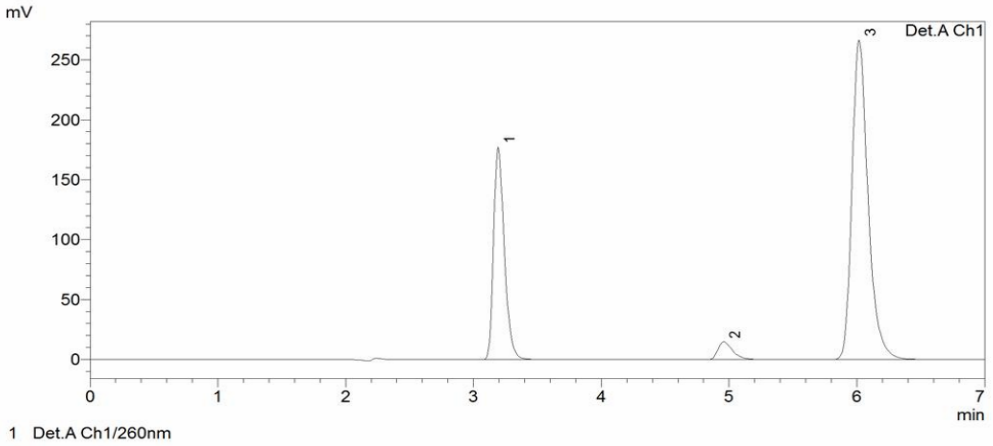

1 Det.A Ch1/260nm

PeakTable

Detector A Ch1 260nm

Ret. Time	Area	Theoretical Plate#	Tailing Factor	Resolution
3.189	1031415	6032.284	1.422	0.000
4.954	118921	8407.109	1.603	9.279
6.013	2330914	10726.382	1.370	4.724

Appendix IV Precission for amlodipine hydrochlorothiazide and losartan (80% of target concentration, Injection No. 1)

==== Shimadzu LCsolution Analysis Report ====

F:\hctz amlo val\7 Rubestness\ACN less\Amlo-Val-Hctz Robustnes 5%less ACN 100% spiked 1.lcd

Acquired by	: Admin
Sample Name	: Amlo-Val-Hctz Robustnes 5%less ACN 100% spiked 1
Sample ID	: Amlo-Val-Hctz Robustnes 5%less
Tray#	: 1
Vail #	: 11
Injection Volume	: 20 uL
Data File Name	: Amlo-Val-Hctz Robustnes 5%less ACN 100% spiked 1.lcd
Method File Name	: Amlo-Val-Hctz.lcm
Batch File Name	: Amlo-Val-Hctz.lcb
Report File Name	: ok ok.lcr
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Data Processed	: 10/26/2015 1:10:24 AM

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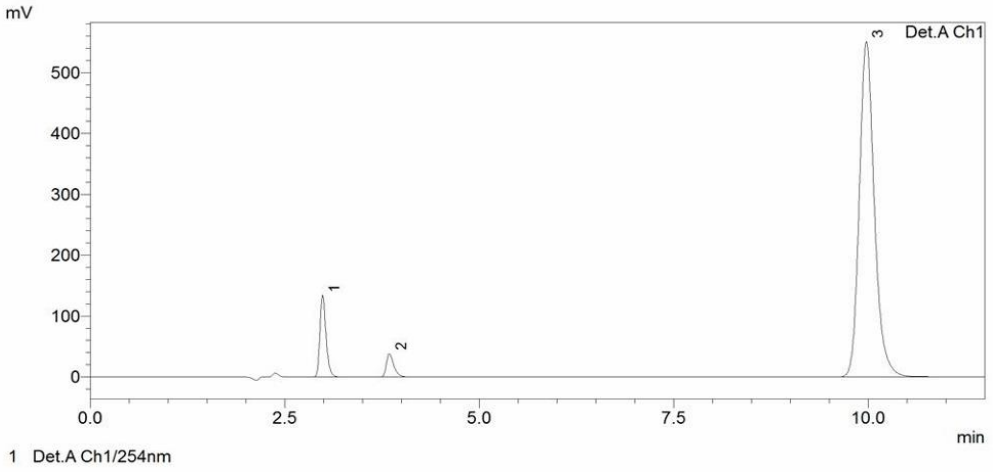

1 Det.A Ch1/254nm

PeakTable

Detector A Ch1 254nm

Ret. Time	Area	Theoretical Plate#	Tailing Factor	Resolution
2.985	740302	5837.964	1.356	0.000
3.843	258565	6859.755	1.537	5.020
9.974	7416662	13066.383	1.236	22.937

Appendix V Robustness for amlodipine valsartan and hydrochlorothiazide (target concentration, Injection No. 1)