ANALYTICAL METHOD DEVELOPMENT FOR THE ESTIMATION OF OLMESARTAN AND AMLODIPINE BISILATE COMBINATION IN TABLET DOSAGE FORM BY USING HPLC METHOD

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Abstract

The development of analytical method for the determination of drugs in bulk, in dosage forms or in body fluids have received attention in recent years because of their importance in quality control, bioavailability and pharmacokinetic study etc. Amlodipine besylate and olmesartan medoxomil both drugs come under the category of antihypertensive drug. This combination is useful for the treatment of hypertention and cardiovascular associated disorder e.g. angina. The aim and objective of the present work is to develop new simple, sensitive, economic and validated RP-HPLC method for the simultaneous estimation of ketorolac and ofloxacine in marketed formulation. Validation of developed Analytical method will be according to ICH guideline. We perform analytical method validation of developed methods i.e. specificity, linearity, accuracy, precision, repeatability, intermediate precision, and interday precision. We can conclude that the HPLC methods for quantitative estimation of pharmaceuticals are fast, less time consuming, reproducible and highly sensitive even microgram of compound can be measured. Performing a through method validation can be a tedious process, but the quality of data generated with the method is directly linked to the quality of this process. Time constraints often do not allow for sufficient method validations. Many researchers have experienced the consequences of invalid methods and realized that the amount of time and resources required to solve problems discovered later exceeds what would have been expended initially if the validation studies had been performed properly.

Key words: Olmesartan, Amlodipine, HPLC, Hypertention, cardiovascular disorder, Solubility.

Introduction

An analytical method details the steps necessary to perform an analysis. This may include preparation of samples, standards and reagents; use of the apparatus; generation of the calibration curve, use of the formulae for the calculation, etc. The objective of validation of an analytical method is to demonstrate that the method is suitable for the intended use.[1] At present several analytical methods are available for analyzing analyte viz. Spectroscopic and Chromatographic, Electrochemical and other Conventional methods etc. Quality of manufactured drug in tablets, solution and emulsion from must be carefully controlled in pharmaceutical industry otherwise the drug can itself affect the therapeutic value. In other pharmaceutical studies, it is important to establish the properties and therapeutic value of a drug before the drug is approved and made available to the patients.[2,3]

Chromatography is a physical separation in which the components of a mixture are separated by differences in their distribution between two phases, one of which is stationary (stationary phase) while the other (mobile phase) moves through it in a definite direction. The substances must interact with the stationary phase to be retained and separated by it. Chromatographic methods are generally classified according to the physical state of the solute carrier phase, which is the mobile phase. The main divisions of chromatography, based on mobile phase, may also be subdivided according to the mechanism of solute interaction with the stationary phase. Two mechanisms, adsorption and partition, are the most commonly encountered for both, solution and gas mobile phase separations. The classifications are represented in the following **figure 1**.

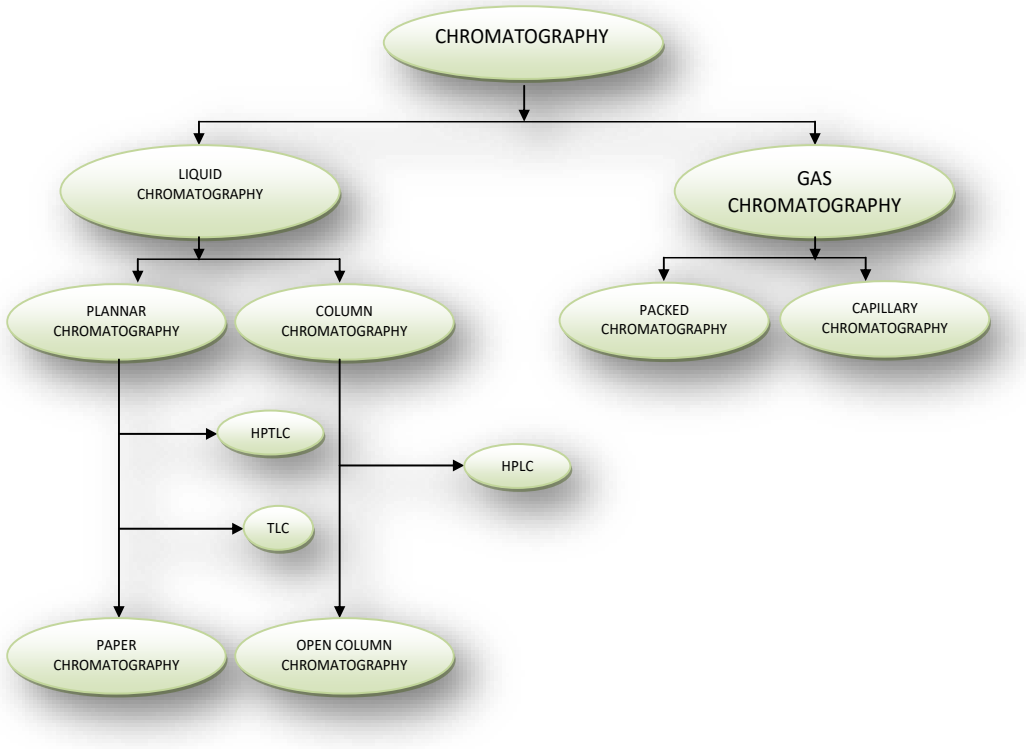


Figure no. 1:- Classification of chromatography, according to Mobile phase and physical apparatus.

The stationary phase is strongly polar in nature (e.g., silica gel), and the mobile phase is non polar (such as n-hexane or tetrahydrofuran). Polar samples are thus retained on the polar surface of the column packing longer than less polar materials. In adsorption mode of liquid chromatography, solutes are retained as a result of the ability of the stationary phase to bond them temporarily to its active surface. The forces involved usually are relatively weak and effective only over short distances. The analytes interact with the stationary phase according to the premise "like likes like". Polar solutes will be retained longest by polar stationary phases and non-polar solutes will be retained best by non-polar stationary phases. In adsorption chromatography the solute molecules are in contact with both the stationary phase and mobile phase, simultaneously. Under these conditions, solutes are said to be in an anisotropic environment. If the mobile phase is liquid, the process is called liquid-solid chromatography (LSC) but when the mobile phase is a gas, the method is called gas-solid chromatography.

The stationary phase is liquid; an inert solid material- such as silica gel, diatomaceous earth, or even the walls of the column itself serves to support a thin layer of liquid which is the effective stationary phase. As the mobile phase containing the solutes passes in close proximity to this stationary phase, retention and separation occurs due to relative solubility of

the analytes in the two fluids as determined by their partition coefficients. If the mobile phase is liquid, it is called Liquid-Liquid Chromatography (LLC) and, if the mobile phase is gas, the process is termed Gas-Liquid Chromatography (GLC). It is based on the principle that opposites attract.

Ion exchange chromatography is used to separate charged analytes and therefore occurs as a result of interaction between a charged solute and oppositely charged, stationary phase. Ion exchange chromatography can be applied to any solute that can acquire a charge in solution. Thus, even carbohydrates, which are largely uncharged below pH 12, can be separated by ion exchange chromatography at a high pH. It is based on the sieving principle. The stationary phase is a polymeric substance containing numerous pores of molecular dimensions. Solutes whose molecular size is sufficiently small leave the mobile phase to diffuse into the pores. Larger molecules that will not be fit into the pores remains in the mobile phase and are not retained. This method is most suited to the separation of mixtures in which the solutes vary considerably in molecular size. The mobile phase in this type may be either liquid or gas.

It is based on the lock and key mechanism, prevalent in biological system. The retention mechanism is very specific, but the technique was very time consuming and more expensive than those employing other retention mechanisms.[8-1] High performance liquid chromatography is basically a highly improved form of column chromatography. It is the most widely used form of chromatography. Most of the drugs in multicomponent dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC method eliminates tedious extraction and isolation procedures.

This provides the constant and continuous flow of the mobile phase through the system. Most of the modern pumps allow controlled mixing of different solvents from different reservoirs. High pressure pumps are needed to force solvents through packed stationary phase beds. Smaller bed particles require higher pressures. There are many advantages to using smaller particles, but they may not be essential for all separations. Many separation problems can be resolved with larger particle packing's that require less pressure. Flow rate stability is another important pump feature that distinguishes pumps. Very stable flow rates are usually not essential for analytical chromatography. However, if the user plans to use a system in size exclusion mode, then there must be a pump which provides an extremely stable

flow rate. An additional feature found on the more elaborate pumps is external electronic control. Although it adds to the expense of the pump, external electronic control is a very desirable feature when automation or electronically controlled gradients are to be run. Alternatively, this becomes an undesirable feature (since it is an unnecessary expense) when using isocratic methods. The degree of flow control also varies with pump expense. It is desirable to have an integrated degassing system, either helium purging, or membrane filtering.

This allows an introduction (injection) of the analytes mixture into the stream of the mobile phase before it enters the column. Most of the modern injectors are auto samplers, which allow programmed injections of different volumes of samples that are withdrawn from the vials in the auto sampler tray. Sample introduction can be accomplished in various ways. The simplest method is to use an injection valve. In more sophisticated LC systems, automatic sampling devices are incorporated where the sample is introduced with the help of auto samplers and microprocessors. In liquid chromatography, liquid samples may be injected directly and solid samples need only be dissolved in an appropriate solvent. It is necessary to remove particles from the sample by filtering over a 5μm filter, or centrifuging, since continuous injections of particulate material will eventually cause blockages in injection devices or columns. Sample sizes may vary widely. The availability of highly sensitive detectors frequently allows use of the small samples which yield the highest column performance. In general, it will be noted that much less sample preparation is required in LC than in GC since unwanted or interfering compounds, or both, may often be extracted, or eliminated, by selective detection.

This is the heart of HPLC system. It actually produces a separation of the analytes in the mixture. A column is the place where the mobile phase is in contact with the stationary phase, forming an interface with enormous surface. Most of the chromatographic development in recent years went toward the design of many different ways to enhance this interfacial contact. Typical HPLC columns are 5, 10, 15 and 25 cm in length and are filled with small diameter (3, 5 or 10 μm) particles. The internal diameter of the columns is usually 4.6 mm; this is considered the best compromise for sample capacity, mobile phase consumption, speed and resolution. However, if pure substances are to be collected (preparative scale) then larger diameter columns may be needed. Packing of the column tubing with small diameter particles requires high skill and specialized equipment. .In general, LC columns are fairly durable and one can expect a long service life unless they

are used in some manner which is intrinsically destructive, for example, with highly acidic or basic eluents, or with continual injections of 'dirty' biological or crude samples. It is wise to inject some test mixture (under fixed conditions) into a column when new, and retain the chromatogram. If questionable results are obtained later, the test mixture can be injected again under specified conditions. The two chromatograms may be compared to establish whether or not the column is still useful.

Olmesartan is a synthetic imidazole derivative and angiotensin II receptor antagonist with antihypertensive activity. Olmesartan selectively binds to the angiotensin type 1 (AT1) receptor subtype in vascular smooth muscle and adrenal gland, thereby competing with angiotensin II for binding to the AT1 receptor. Olmesartan belongs to the angiotensin II receptor blocker (ARB) family of drugs. ARBs selectively bind to angiotensin receptor 1 (AT1) and prevent the protein angiotensin II from binding and exerting its hypertensive effects. As the principal pressor agent of the renin-angiotensin system, angiotensin II causes vasoconstriction, stimulation of synthesis and release of aldosterone, cardiac stimulation and renal reabsorption of sodium. Olmesartan blocks the vasoconstrictor effects of angiotensin II by selectively blocking the binding of angiotensin II to the AT1 receptor in vascular smooth muscle. Its action is, therefore, independent of the pathways for angiotensin II synthesis. Overall, olmesartan's physiologic effects lead to reduced blood pressure, lower aldosterone levels, reduced cardiac activity, and increased excretion of sodium. Olmesartan also effects on the renin-angiotensin aldosterone system (RAAS) plays an important role in hemostasis and regulation of kidney, vascular, and cardiac functions. Pharmacological blockade of RAAS via AT1 receptor blockade inhibits negative regulatory feedback within RAAS, which is a contributing factor to the pathogenesis and progression of cardiovascular disease, heart failure, and renal disease.

Materials and Method

1. Methods for Determination of Solubility

Solubility of Amlodipine besylate and olmesartan medoxomil was performed in different solvents as described in Indian Pharmacopoeia.

2 Method for Determination of λ_{max} of Drugs

Standard solution (10µg/ml) of pure Amlodipine besylate and olmesartan medoxomil was prepared. The pure drug solutions were scanned on UV-spectrophotometer from 200- 400 nm.

Also an overlain spectrum of pure Amlodipine besylate and olmesartan medoxomil drugs was obtained to select isosbestic point for further procedure on HPLC.

5.2.3 Method for Preparation of Standard Stock Solutions

10 mg of Amlodipine besylate was weighed accurately and transferred to a 10ml volumetric flask, and the volume was adjusted to the mark with the mobile phase, to give a stock solution of 1000ppm and 10 mg of olmesartan medoxomil was weighed accurately and transferred to a 10ml volumetric flask, and the volume was adjusted to the mark with the mobile phase methanol to give a stock solution of 1000ppm

5.2.4 Method for Preparation of Working Standard Solutions

From stock solutions of Amlodipine besylate 1 ml was taken and diluted up to 10 ml in the separate volumetric flask gives 100ppm solution, from this 100ppm solution, 1, 2, 3, 4 and 8 ml was taken and diluted up to 10 ml in the separate volumetric flask gives standard Amlodipine besylate solutions of 10, 20, 30, 40 and 80 ppm concentration.

From stock solutions of olmesartan medoxomil 1 ml was taken and diluted up to 10 ml in the separate volumetric flask gives 100ppm solution, from this 100ppm solution, 1, 2, 3, 4 and 8 ml was taken and diluted up to 10 ml in the separate volumetric flask gives standard olmesartan medoxomil solutions of 10, 20, 30, 40 and 80 ppm concentration.

5.2.5 Preparation of Phosphate Buffer Solution pH = 3.0 (I.P.)

Dissolve 1.36 g of potassium dihydrogen orthophosphate and 2 ml of triethylamine in 800ml of water, adjust the pH to 3.0 with o-phosphoric acid and add sufficient water to produce 1000 ml.

5.2.6 Preparation of Mobile Phase

Mobile phase prepare by mixing Buffer prepared in water (HPLC grade) and acetone (HPLC grade) in selected proportion (90:10) and maintained pH 3.0 by ortho-phosphoric acid. Prepared Mobile phase taken separately filtered through membrane nylon filters of size 0.45 µ, to the filtered solution and the mixed solution then sonicated for 15 minutes.

5.2.7 Method for Preparation of the Calibration Curves of the Drug

Each of the standard drug solutions were injected 3 times and the mean peak area of drug was calculated and plotted against the concentration of the drug. The regression equation was found out by using this curve. A typical chromatogram and the calibration curve were obtained.

5.2.8 Method for Analysis of Formulation

Separately weighed 10 tables of marketed formulation were triturated in mortar pastle. Equivalent to 10 mg of Amlodipine besylate was taken in 10 volumetric flask and volume was made up to 10 ml with solvent to obtain concentration of 1000µg/ml. Resultant solution was filtered through Whatmann filter paper. 1 ml of filtrate was taken in 10 ml volumetric flask and volume was made up to 10 ml with solvent to obtain concentration of 100µg/ml. Further 5 ml of this solution was taken and diluted up to 10 ml obtain final concentration of 50µg/ml and injected in to HPLC.

5.3 Methods of Validation

5.3.1 Linearity

Linearity of analytical procedure is its ability (within a given range) to obtain test, which are directly proportional to absorbance of analyte in the sample. The calibration plot was contracted after analysis of five different concentrations and absorbances for each concentration was recorded three times, and mean absorbance was calculated. The regression equation and correlation coefficient of curve and the standard curve of the drug is calculated.

5.3.2 Accuracy

Recovery studies were performed to validate the accuracy of developed method. To pre-analyzed sample solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and then its recovery was analyzed and statistical validation of recovery studied.

Recovery: The extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted

samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery.

5.3.3 Precision

5.3.3.1 Repeatability

Standard dilutions were prepared and three replicates of each dilution were analyzed in same day for repeatability and results were subjected to statistical analysis. Standard dilutions were prepared and three replicates of each dilution were analyzed in different days and by different analysts. Statistical analysis was carried out.

(A) Intermediate Precision: **(a) Day to Day (b) Analyst to Analyst**

The intermediate precision expresses with in laboratories variation: different days, different analysts, different equipment etc. The standard dilution was prepared and three replicate of each dilution were analyzed by different analysts for all the developed methods. The statistical analysis method was carried out and the data is presented in the table.

5.3.4 Range

The range of method can be defined as the lower and upper concentrations for which the analytical method has adequate accuracy, precision, and linearity. The range of concentrations examined will depend on the type of method and its use. For a major component assay, concentrations of standards should be measured at or near the expected target measurement level.

5.3.4.1 Limit of detection (LOD)

The lowest concentration of an analyte that the analytical procedure can reliably differentiate from background noise called limit of detection.

5.3.4.2 Lower limit of quantification (LLOQ)

The lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy called lower limit of quantification.

5.3.4.3 Upper limit of quantification (ULOQ)

The highest amount of an analyte in a sample that can be quantitatively determined with precision and accuracy called upper limit of quantification.

5.3.5 Specificity

Specificity can be defined as the ability to measure accurately the concentration of an analyte in the presence of all other sample materials the determination of method specificity can be achieved in two ways, first and most desirable, all potential interfering compounds can be tested to demonstrate their separation from the peak (s) of interest with a specified resolution (usually RS ≥ 2) A second method for achieving a specificity is the use of selective detectors especially for co-eluting compounds. For example a selective detectors (e.g. electrochemical and radioactivity) will respond some compounds but not to others.

5.3.6 Ruggedness

Method ruggedness is defined as the reproducibility of results when the method is performed under actual use conditions. This includes different analysts, laboratories, columns, instruments, source of reagents, chemicals, solvent and so on.

5.3.7 Robustness

As per ICH norms, small, but deliberate variations, by altering the pH and / or concentration of the mobile phase were made to check the method capacity to remain unaffected. The change was made in the ratio of mobile phase, pH of mobile phase.

5.3.8 System Suitability

It can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The USP defines parameters that can be used to determine the system suitability prior to analysis. These parameters include plate No. (n), tailing factor, *k* and / or α , resolution (Rs), and relative standard deviation (RSD) of peak height or peak area or repetitive injections.

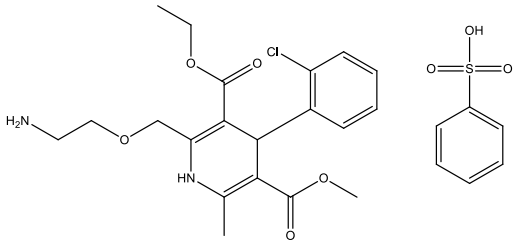
5.3.9 Selectivity

Selectivity is the ability of the analytical method to measure and differentiate the analytes in the presence of components that may be expected to be present in it. These could include impurities, degradants, or matrix components. Selectivity should be ensured at the lower limit of quantification (LLOQ).

Results and Discussion

1 Drug identification

6.1.1 Amlodipine Besylate

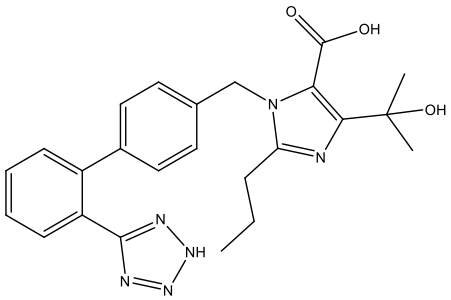


IUPAC name: Benzenesulfonic acid; 3-ethyl 5-methyl 2-((2-aminoethoxy)methyl)-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate

Table No: Physical observation of Amlodipine

S. No.	Particulars	Reported	Observed
1.	Appearance	White crystalline	White amorphous
2.	Melting point	199-201 ^{O}C	200-202 ^{O}C

6.1.2 Olmesartan



IUPAC Name: 5-(2-hydroxypropan-2-yl)-2-propyl-3-[[4-[2-(2*H*-tetrazol-5-yl)phenyl]phenyl] methyl] imidazole-4-carboxylic acid

Table No 5: Physical observation of Olmesartan

S. No.	Particulars	Reported	Observed
1.	Appearance	White to light yellow amorphous	White light yellow amorphous
2.	Melting point	175 - 177 ^{O}C	175-176 ^{O}C

6.2 Method Development for Estimation of Amlodipine and Olmesartan on RP-HPLC

6.2.1 Wavelength selection for Amlodipine on UV spectrophotometer

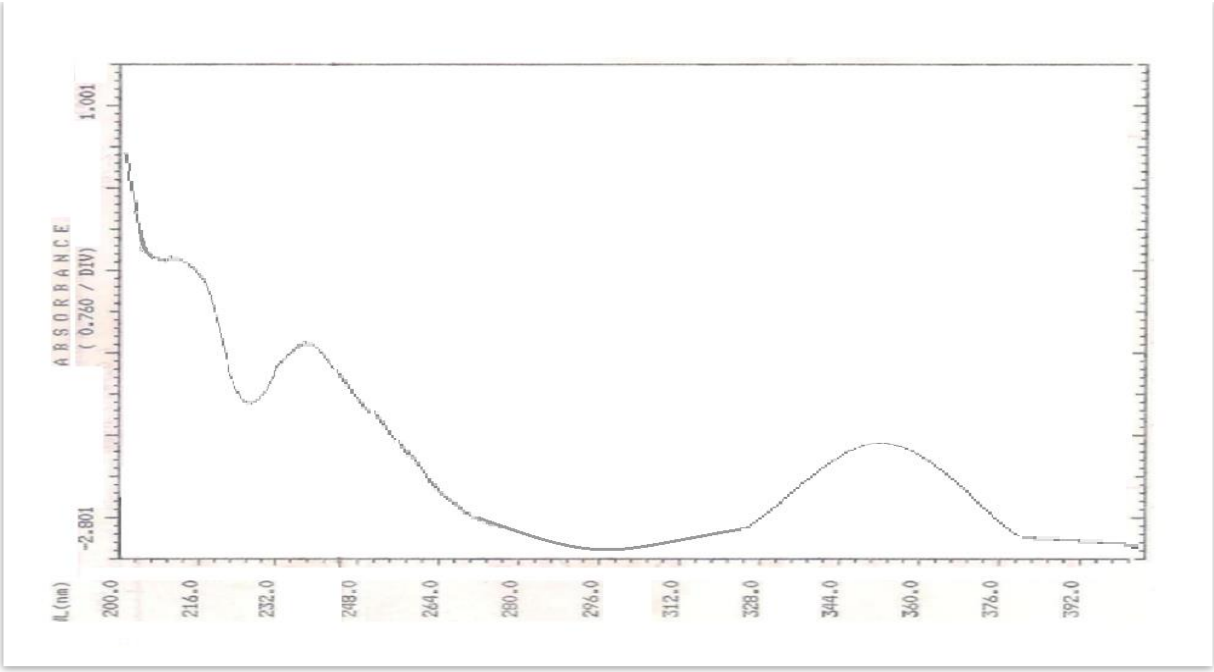


Fig. No. 5: UV Absorption Spectra of Amlodipine

6.2.2 Wavelength selection for Olmesartan on UV spectrophotometer

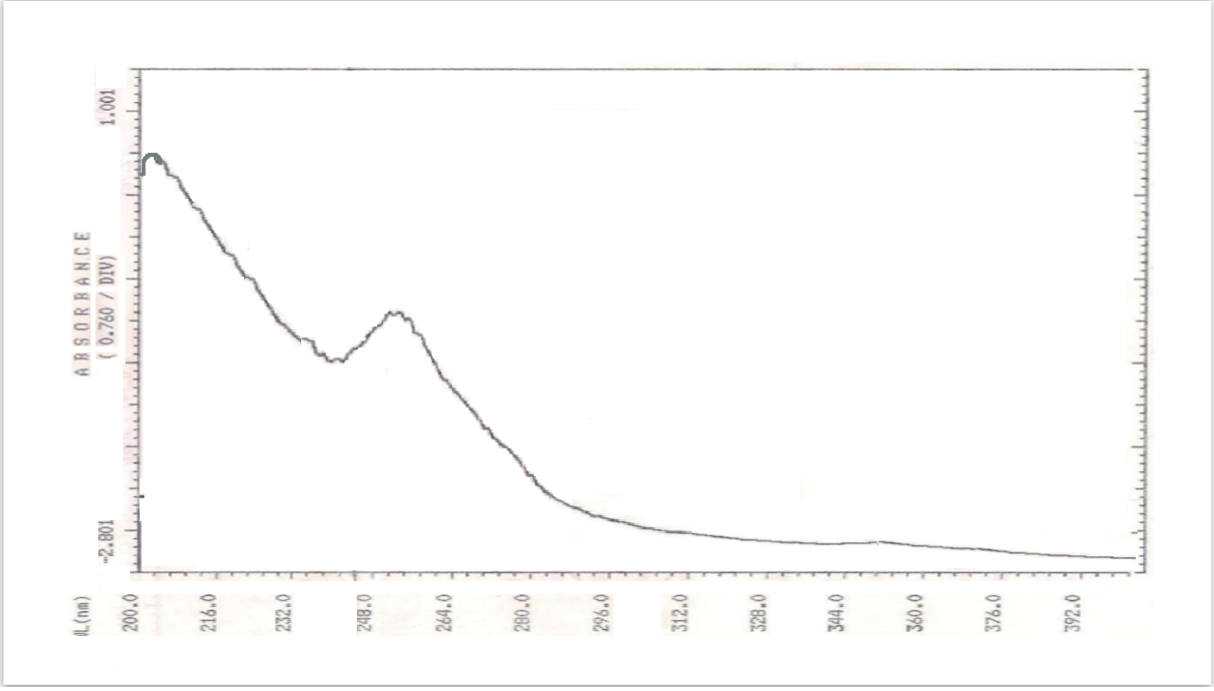


Fig. No. 6: Absorption Spectra of Olmesartan

6.2.3 Overlain UV spectrogram of Amlodipine and Olmesartan

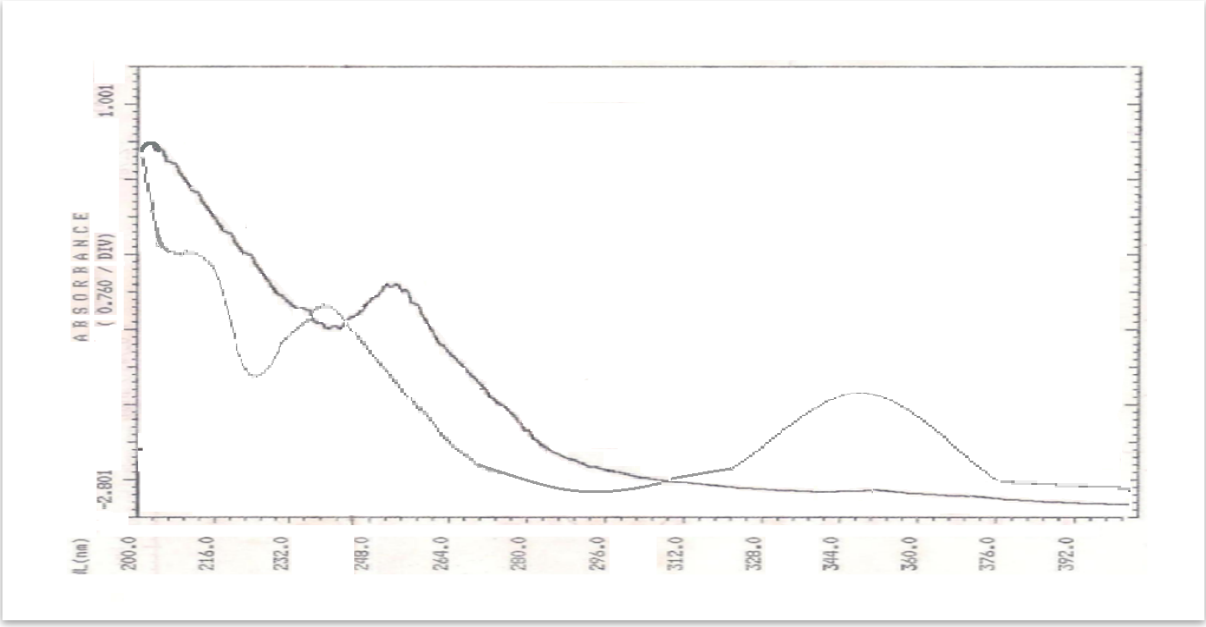


Fig. No. 7: Overlain UV spectrogram of Amlodipine and Olmesartan

Result: The λ_{max} of Amlodipine was found at 239 nm. The λ_{max} of Olmesartan was found to be 256 nm. Three isosbestic points were found in overlain spectra of Amlodipine and Olmesartan that were 235nm, 242nm, and 308.5 nm. Selected wave length (isosbestic point) was 235nm for further studies.

6.2.4 Selection of mobile phase

Table no. 6: Selection of Mobile phase, pH and flow rate

Mobile phase	Ratio	Flow rate (ml/min)	Conclusion
Acetonitrile:Buffer	50:50	0.8 & 1.0	Asymmetric peaks, poor resolution
Buffer: Methanol: ACN	65:25:10	1.0 & 1.2	Good resolution and retention time, but Amlodipine has more asymmetric peaks
Buffer:Methanol	50:50	1.0	Poor resolution and long retention time
Phosphate Buffer:Methanol	80:20	1.2	Shorter retention time for Olmesartan
Phosphate Buffer:Methanol	90:10	1.0	Better resolution and retention time but Asymmetric peaks and tailing found
Phosphate Buffer:Acetone	90:10	1.0	Good resolution and retention time, Asymmetric peaks

Table No. 7: Retention time of Working Standards

Peak	Retention time	Area	Peak Purity	Height

Olmesartan	13.106	237143.787	999	7321.9297
Amlodipine	14.243	257441.011	999	8021.9768

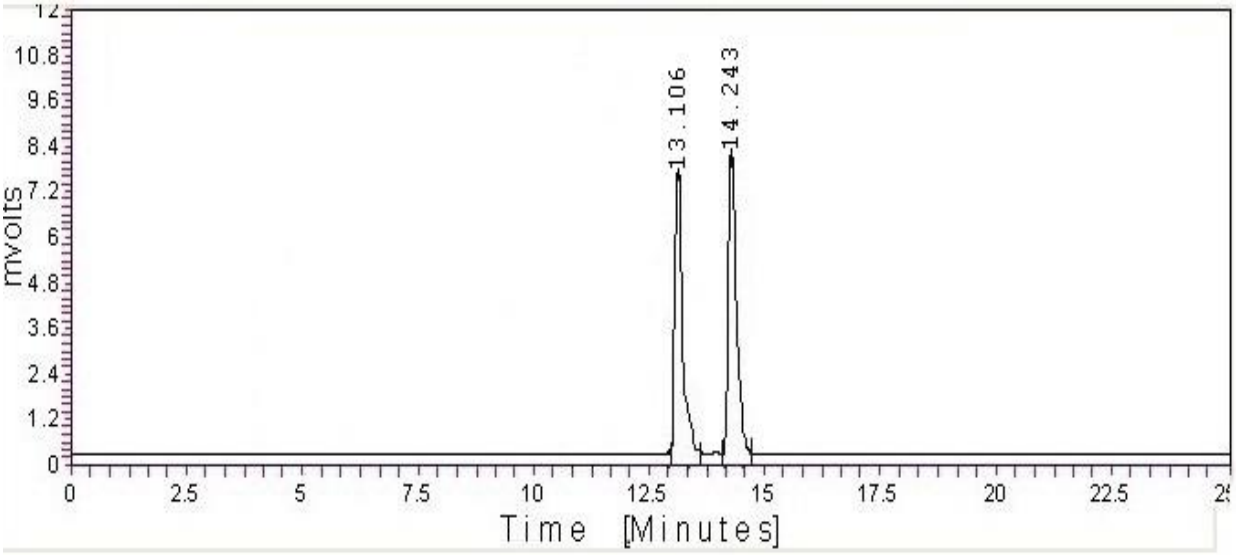


Fig. no. 8: HPLC chromatogram of Amlodipine and Olmesartan

6.3 Assay of Formulation

Table no. 8: Assay of Amlodipine and Olmesartan in marketed tablet

Brand Name	Omten-AM			
	Olmesartan	% Assay	Amlodipine	% Assay
Label Claim (mg)	20 mg/ml	99.9	5 mg/ml	99.9
	20 mg/ml	99.8	5 mg/ml	99.8
	20 mg/ml	99.7	5 mg/ml	99.5
	20 mg/ml	99.9	5 mg/ml	99.9
	20 mg/ml	99.8	5 mg/ml	99.4
Mean	0	99.82	0	99.7
SD	0	0.083666003	0	0.234520788
%RSD	0	0.083816873	0	0.235226467

6.4 Method Validation of Developed Method

6.4.1 System Suitability

Table No. 9: Description of System suitability

S. no.	Parameters	Olmesartan	Amlodipine
1.	Resolution (Rs)	6.7243	6.3458
2.	Capacity Factor (k´)	4.567	4.348
3.	Theoretical Plate	385416.4571	445516.6657
4.	HETP	0.13202	0.1131
5.	Tailing Factor	1.0672	1.0719
6.	Retention time(RT)	13.103	14.201
7.	Asymmetry	1.041	1.105

During mobile phase optimization and considering the system suitability parameters like Rt, Tailing factor, No. of theoretical plates and HETP, the mobile phase phosphate buffer: acetone (90:10), pH was 3.0 at λmax 235nm was found satisfactory. After mobile phase selection, effect of pH and flow rate was observed. It was found that pH = 3 and 1.0 ml/min. is suitable for the drug.

6.4.1.1 Chromatographic conditions

- Column : C_{18} Column (25×0.46cm, i.d,5 µm)
- Column Temperature : 25^0 C
- Flow rate : 1.0 ml/min
- Pump mode : Isocratic
- Injection Volume : 20µl
- Run Time : 20 Min
- pH : 3.0
- Wavelength used : 303.5 nm
- Mobile Phase : Phosphate buffer: Acetone (90:10)

6.4.2 Specificity

Table no. 10: Specificity representation

S. No.	Peak name	Retention Time
1	Diluent	No peaks are observed at retention time of main peak
2	Placebo	No peaks are observed at the retention time of main peak

4	Main Peaks	13.103, 14.201

The chromatograms of blank and mixture drug are given below:

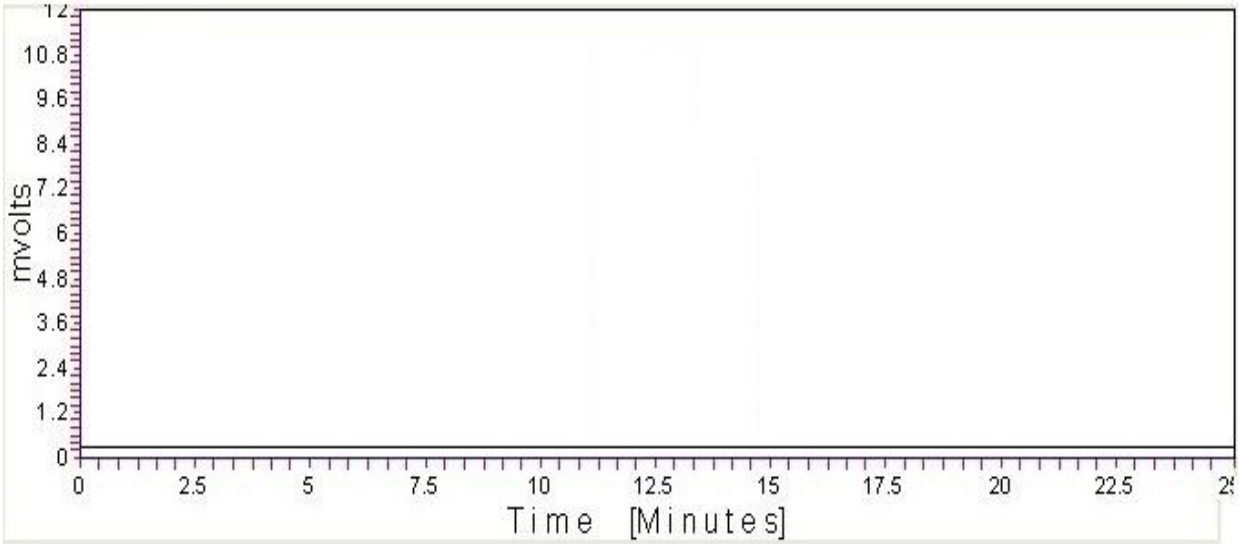


Fig. no.9: Chromatogram of Blank (mobile phase)

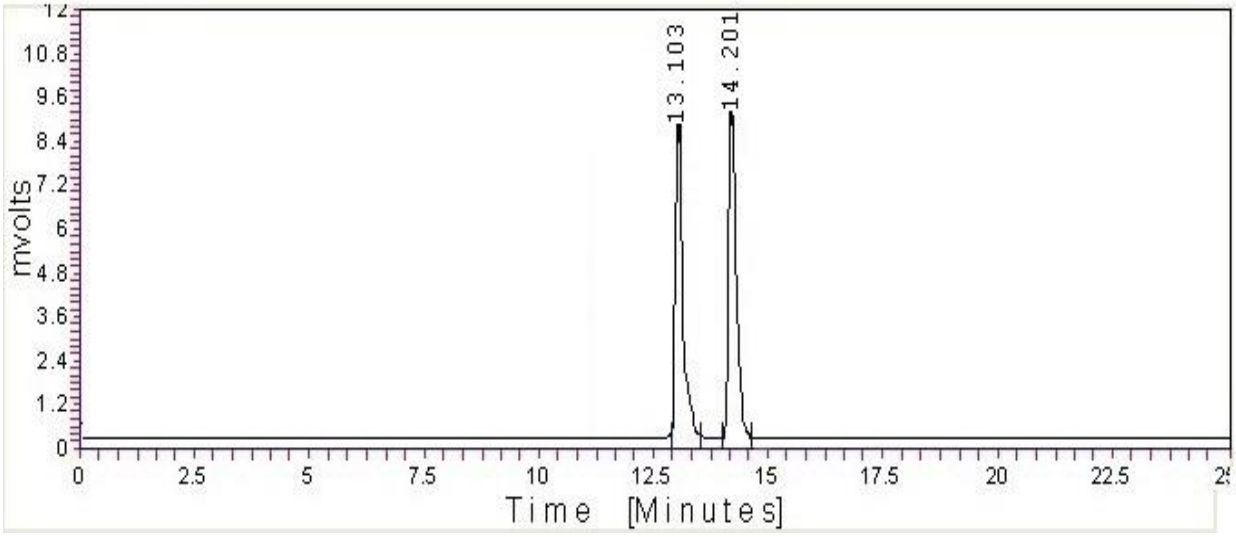


Fig. no.10: Chromatogram of mixture of both standard drugs

6.4.3 Linearity and Calibration curve (10, 20, 30, 40 and 80 ppm)

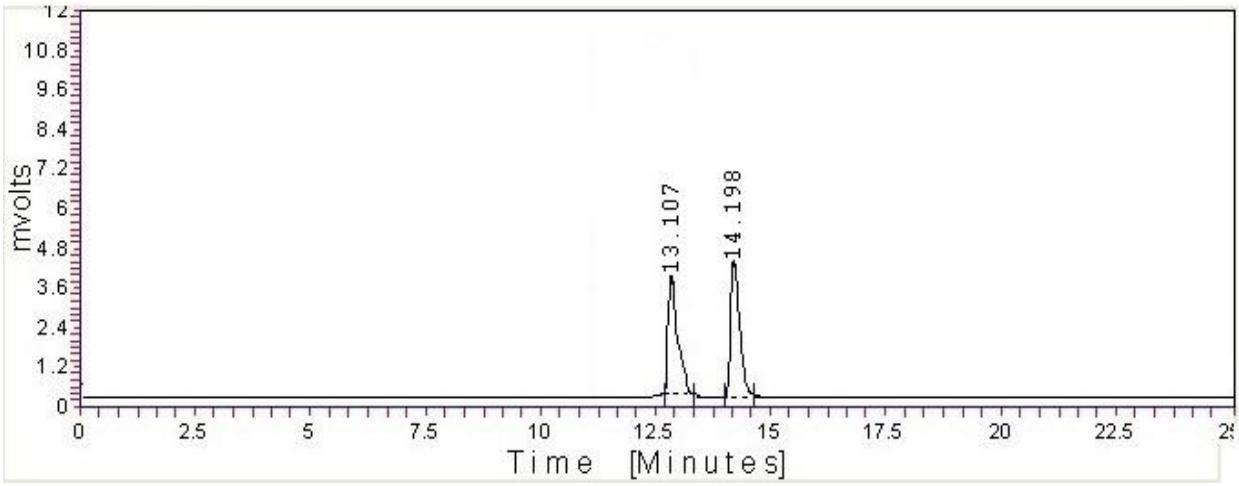


Fig. no. 11: Chromatogram of mixture of both standard drugs at 10ppm

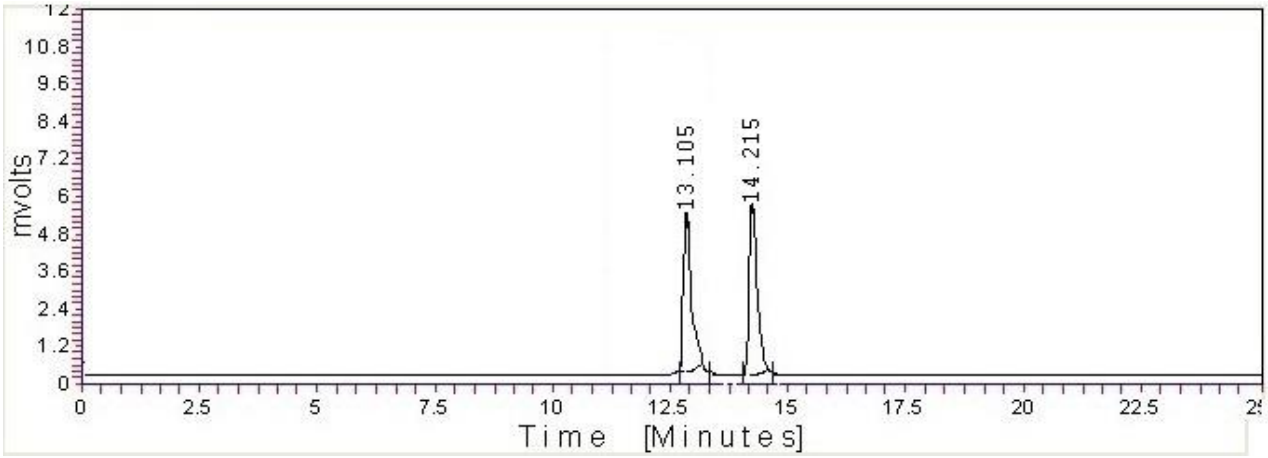


Fig.no. 12: Chromatogram of of mixture of both standard drugs 20 ppm

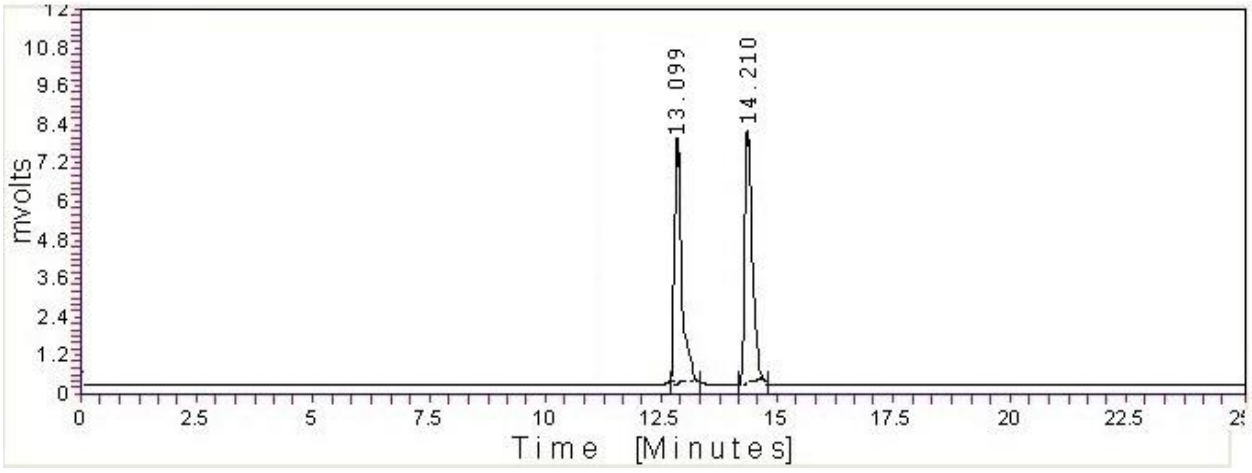


Fig. no.13: Chromatogram of mixture of both standard drugs 30 ppm

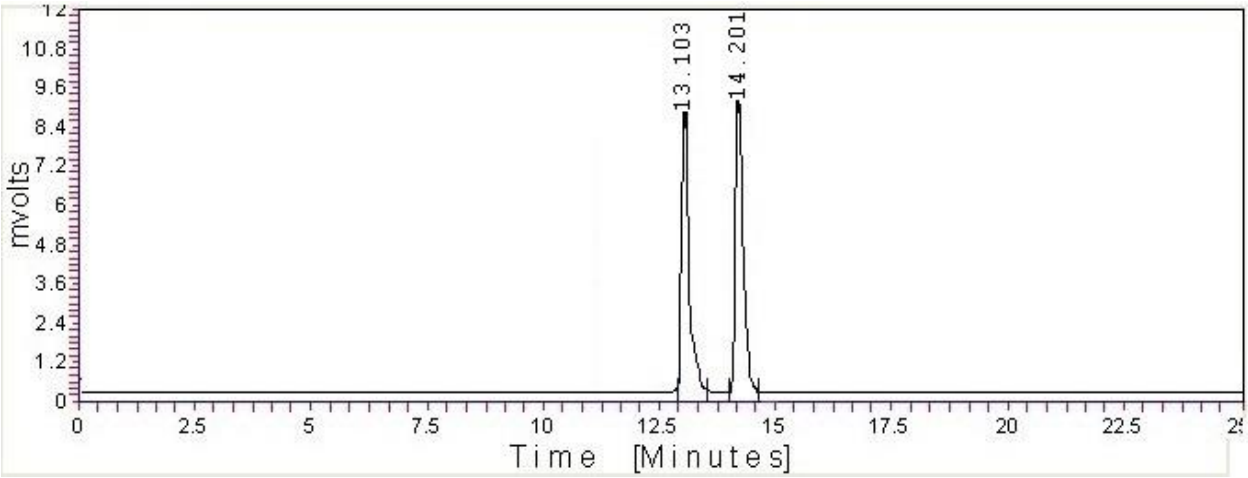


Fig. no. 14: Chromatogram of mixture of both standard drugs 40ppm

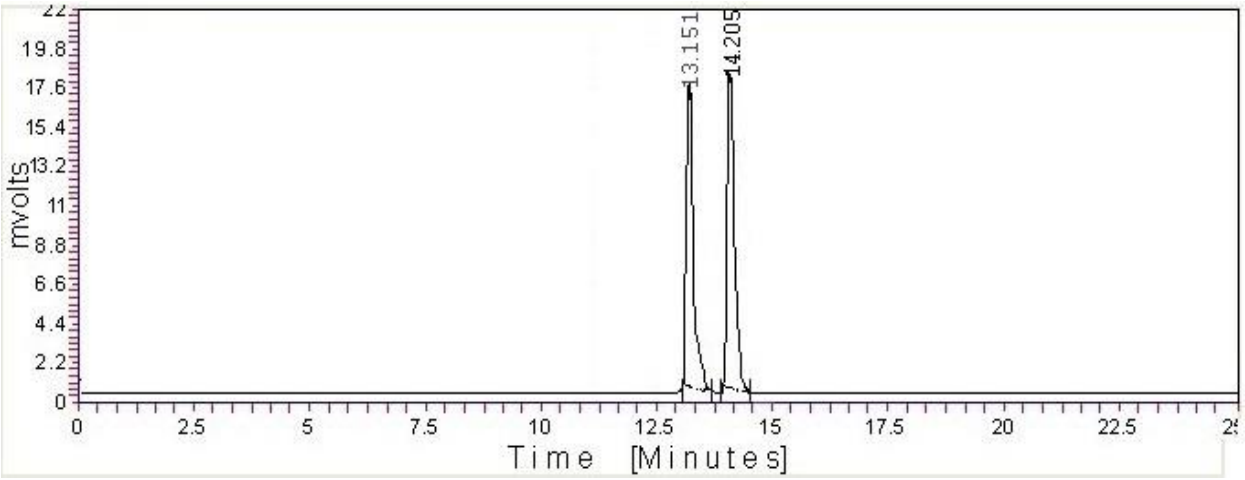


Fig. no. 15: Chromatogram of mixture of both standard drugs 80ppm

Table no.11: Peak areas of different concentrations of Olmesartan

Rep.	10 µg/ml	20 µg/ml	30 µg/ml	40 µg/ml	80 µg/ml
1	144576.5445	249063.5545	359132.5469	437763.5660	871132.6467
2	144523.4455	249079.7111	359126.8722	437783.8456	871156.6840
3	144540.5126	249111.2313	359111.2138	437770.7595	871145.6162
Mean	144546.8342	249084.8323	359123.5443	437772.7237	871144.9823
S. D.	27.10808	24.24746	11.04905	10.28149	12.03118
R.S.D%	0.0187538	0.0097346	0.0030767	0.0023485	0.0013811
LOQ	0.0262243	0.0234596	0.0106889	0.0099463	0.0116389
LOD	0.0086540	0.0077408	0.0035273	0.0032823	0.0038409

Table no. 12: Peak areas of different concentrations of Amlodipine

Rep.	10 µg/ml	20 µg/ml	30 µg/ml	40 µg/ml	80 µg/ml
1	199048.2147	297568.6231	409075.4562	494590.1424	984140.4522
2	199052.4674	297558.4875	409065.6776	494586.6315	984149.2598
3	199056.3035	297563.8147	409080.6122	494582.4663	984156.2451
Mean	199052.3285	297563.6418	409073.9153	494586.4134	984148.6524
S.D.	4.0461876	5.0700124	7.5855963	3.8426948	7.9139531
R.S.D%	0.0020327	0.0017038	0.0018543	0.0007769	0.0008041
LOQ	0.00359214	0.00450108	0.00673437	0.00341148	0.00702588
LOD	0.00118541	0.00148535	0.00222234	0.00112579	0.00231854

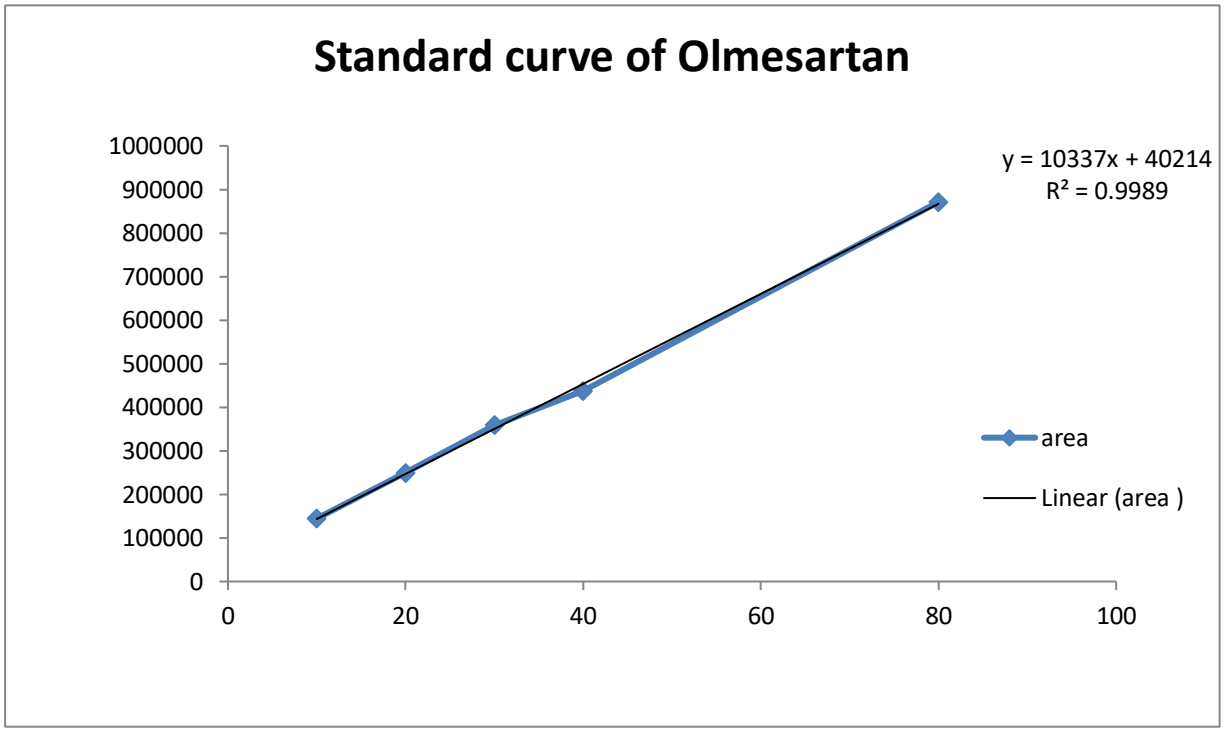


Fig. no. 16: Calibration curve of Olmesartan

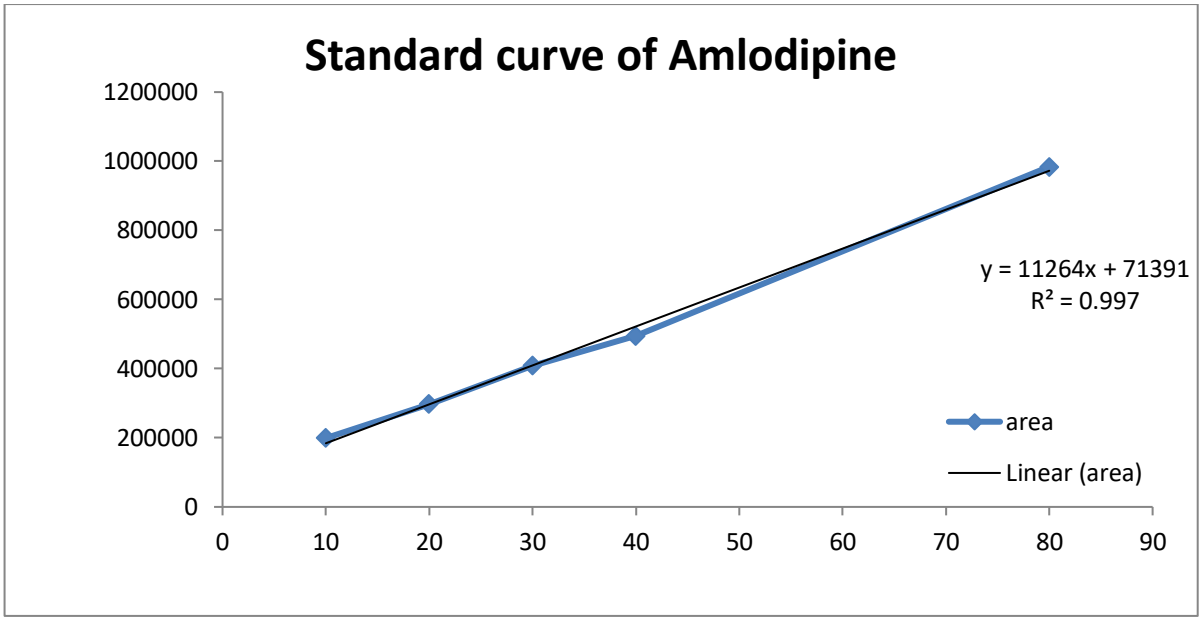


Fig. no. 17: Calibration curve of Amlodipine

6.4.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)

Table No. 13: LOD & LOQ for Olmesartan and Amlodipine

Sample Name	LOD	LOQ
Olmesartan	0.0054091	0.0163916
Amlodipine	0.0016675	0.0050529

6.4.5 Accuracy

The accuracy of the method was done by addition of standard drug solution to pre-analyzed sample solution at three different levels 80, 100 and 120 %. Mean percentage recovery was determined. % recovery was calculated by the given formula:

% Recovery =Amount recover / Total present amount × 100

Table no.14: Recovery Studies and Statistical Validation for Accuracy of Formulation

Statistical Validation	80%		100%		120%	
Recovery (%)	Olmesartan	Amlodipine	Olmesartan	Amlodipine	Olmesartan	Amlodipine
Amount Present	20	5	20	5	20	5
	20	5	20	5	20	5
	20	5	20	5	20	5

Amount of Std. Added	16	4	20	5	24	6
	16	4	20	5	24	6
	16	4	20	5	24	6
Amount Recovered	15.986	3.973	19.899	4.956	23.971	5.961
	15.899	3.997	19.997	4.992	23.864	5.979
	15.959	3.996	19.896	4.971	23.939	6.000
% Recovery	99.91	99.32	99.495	99.12	99.88	99.35
	99.37	99.92	99.985	99.84	99.435	99.65
	99.745	99.89	99.48	99.423	99.745	100
Mean Recovery	99.675	99.71	99.65333	99.461	99.686667	99.66667
SD	0.276722	0.338083	0.287329	0.361501	0.228163	0.325320
%RSD	0.277624	0.339066	0.2883292	0.363461	0.228880	0.326408

6 Precision

The precision of the method was established by caring out analysis of the compound based on intra-day and inter-day analysis. Precision was calculated by relative standard deviation (%RSD) known as percentage Coefficient of Variance (%CV), using the formula:

%RSD = Standard deviation / Mean × 100

Table no.15: Intra-day and Inter-day precision data

Intra-day Precision			**Inter-day Precision**		
Period of Time	**% Label Claim**		**Period of Time**	**% Label Claim**	
	Olmesartan	**Amlodipine**		**Olmesartan**	**Amlodipine**
After 1hr	99.6	99.7	First day	99.8	99.9
After2hr	99.8	99.7	Second day	98.9	99.8
After3hr	98.7	99.8	Third day	98.6	99.9
After4hr	98.9	99.6			
After5hr	99.7	99.9			
After6hr	99.8	99.7			
Mean	99.41666667	99.7333333	**Mean**	99.1	99.8666667

SD	0.487510684	0.10327956	**SD**	0.6244998	0.057735027
% RSD	0.490371182	0.103555709	**% RSD**	0.630171342	0.057812109

Table no. 16: Analyst to Analyst precision

	Olmesartan			**Amlodipine**		
	Label claim Analyst mg	**Amount found mg**	**Label claim (%)**	**Label claim Analyst mg**	**Amount found mg**	**Label claim (%)**
1	20	19.897	99.485	5	4.967	99.344
2	20	19.998	99.99	5	4.978	99.55
3	20	19.991	99.955	5	4.982	99.64
Mean	20	19.962	99.81	5	4.976	99.511
SD	0	0.0564	0.282001773	0	0.0078	0.15174101
% RSD	0	0.2825	0.282538596	0	0.1567	0.15248667

6.4.7 Ruggedness and Robustness

6.4.7.1 Effect of pH on method

Table no. 17: Effect of pH on Standard Olmesartan drug

Standard Olmesartan drug				
pH	**Rt**	**Area**	**Tailing**	**Plate count**
2.8	13.107	251278.5864	1.121	214181.654
3.2	13.105	251289.3169	1.139	214177.744
Mean	13.106	251283.9517	1.13	214179.699
S.D.	0.001414214	7.587609316	0.012727922	2.764787514
%R.S.D.	0.010790581	0.003019536	1.126364784	0.001290873

Table no.18: Effect of pH on Olmesartan in Formulation

Olmesartan in Formulation				
pH	**Rt**	**Area**	**Tailing**	**Plate count**
2.8	13.105	251264.8983	1.119	214179.614
3.2	13.103	251272.6774	1.126	214183.654
Mean	13.104	251268.7879	1.1225	214181.634
S.D.	0.001414214	5.500654362	0.004949747	2.856711396
%RSD	0.010792228	0.002189151	0.440957458	0.00133378

Table no. 19: Effect of pH on Standard Amlodipine drug

Standard Amlodipine drug				
pH	**Rt**	**Area**	**Tailing**	**Plate count**
2.8	14.181	239573.8691	1.122	214180.464
3.2	14.187	239591.7904	1.141	214188.684
Mean	14.184	239582.8298	1.1315	214184.574
S.D.	0.004242641	12.67227276	0.013435029	5.812417741
%RSD	0.029911454	0.005289308	1.187364458	0.002713742

Table no. 20: Effect of pH on Amlodipine drug in Formulation

Amlodipine drug in Formulation				
pH	**Rt**	**Area**	**Tailing**	**Plate count**
2.8	14.184	239566.3684	1.121	214178.994
3.2	14.199	239581.7582	1.109	214184.164
Mean	14.1915	239574.0633	1.115	214181.579
S.D.	0.010606602	10.88223194	0.008485281	3.655742059
%RSD	0.074739116	0.004542325	0.761011782	0.001706842

6.4.7.2 Effect of temperature

Table no. 21: Effect of temperature on Standard Olmesartan drug

Standard Olmesartan drug				
Temp. °C	**Rt**	**Area**	**Tailing**	**Plate count**
25°C	13.101	251258.2425	1.111	214078.794

35°C	13.114	251266.5771	1.108	214086.664
Mean	13.1075	251262.4098	1.1095	214082.729
S.D.	0.009192388	5.893452178	0.00212132	5.564930368
%R.S.D.	0.070130751	0.002345537	0.191196065	0.00259943

Table no. 22: Effect of temperature on Olmesartan in Formultion

Olmesartan in Formultion				
Temp. °C	**Rt**	**Area**	**Tailing**	**Plate count**
25°C	13.105	251255.4274	1.109	214188.764
35°C	13.103	251249.2263	1.203	214179.674
Mean	13.104	251252.3269	1.156	214184.219
S.D.	0.001414214	4.384839861	0.066468037	6.427600641
%RSD	0.010792228	0.001745194	5.749830228	0.003000968

Table no. 23: Effect of temperature on Standard Amlodipine drug

Standard Amlodipine drug				
Temp. °C	**Rt**	**Area**	**Tailing**	**Plate count**
25°C	14.198	239782.6617	1.112	214081.654
35°C	14.201	239796.5603	1.114	214088.394
Mean	14.1995	239789.611	1.113	214085.024
S.D.	0.00212132	9.827794309	0.001414214	4.765899705
%RSD	0.014939402	0.004098507	0.127063213	0.002226171

Table no. 24: Effect of temperature on Amlodipine drug in Formulation

Amlodipine drug in Formulation				
Temp. °C	**Rt**	**Area**	**Tailing**	**Plate count**
25°C	14.183	239553.8691	1.112	214187.574
35°C	14.18	239581.7904	1.111	214181.844
Mean	14.1815	239567.8298	1.1115	214184.709
S.D.	0.00212132	19.74334102	0.000707107	4.051721856
%RSD	0.014958364	0.008241232	0.063617344	0.001891695

6.4.7.3 Effect of flow rate

Table no. 25: Effect of flow rate on Standard Olmesartan drug

Standard Olmesartan drug				
Flow rate ml/min	**Rt**	**Area**	**Tailing**	**Plate count**
1	13.102	251254.2292	1.204	214186.654
1.3	13.11	251239.2592	1.18	214195.474
Mean	13.106	251246.7442	1.192	214191.064
S.D.	0.005656854	10.58538851	0.016970563	6.23668181
%R.S.D.	0.043162325	0.004213145	1.423704929	0.002911738

Table no. 26: Effect of flow rate on Olmesartan in Formultion

Olmesartan in Formultion				
Flow rate ml/min	**Rt**	**Area**	**Tailing**	**Plate count**
1	13.104	251287.4606	1.172	214181.654
1.3	13.112	251301.5395	1.162	214176.214
Mean	13.108	251294.5001	1.167	214178.934
S.D.	0.005656854	9.955285662	0.007071068	3.84666089
%RSD	0.043155739	0.003961601	0.605918407	0.001796003

Table no.27: Effect of flow rate on Standard Amlodipine drug

Standard Amlodipine drug				
Flow rate ml/min	**Rt**	**Area**	**Tailing**	**Plate count**
1	14.185	239556.3496	1.198	214191.864
1.3	14.204	239596.2704	1.13	214179.344
Mean	14.1945	239576.31	1.164	214185.604
S.D.	0.013435029	28.22826799	0.048083261	8.8529769
%RSD	0.094649539	0.011782579	4.130864357	0.00413332

Table no. 28: Effect of flow rate on Amlodipine drug in Formulation

Amlodipine drug in Formulation				
Flow rate ml/min	**Rt**	**Area**	**Tailing**	**Plate count**
1	14.078	239563.8691	1.174	214188.654
1.3	14.099	239591.7904	1.178	214166.214
Mean	14.0885	239577.8298	1.176	214177.434
S.D.	0.014849242	19.74334064	0.002828427	15.86747599
%RSD	0.10539974	0.008240888	0.240512511	0.007408566

Discussion

Amlodipine besylate and olmesartan medoxomil are a white and white to light yellow amaurphus powder melts on 202-202 ^{o}C and 175-176 ^{o}C respectively. UV scanning between 200-400 nm λ_{max} of Amlodipine was found at 239nm and λ_{max} of olmesartan was found to be 256 nm. Three isosbestic points were found in overlain spectra of Amlodipine and Olmesartan that were 235nm, 242nm, and 308.5 nm. 235 nm was selected for further studies. Phosphate buffer:Acetone in 90:10 ratios were selected as mobile phase, flow rate 1.0 ml/min. Retention time of Olmesartan and Amlodipine was found at 13.106 and 14.243 min respectively.

Marketed formulation **Ometen - AM** tablets was used to estimate Olmesartan and Amlodipine by HPLC. Assay result data was excellent, more 99 % drugs estimated. C_{18} Column (25×0.46cm, i.d,5 µm) was used in isocratic mode.

Method was validated on different parameters like **Linearity and Calibration curve** (y = 11264x + 71391 (R^2= 0.997) for Amlodipine and y = 10337x + 40214 (R^2= 0.998) for Olmesartan), **LOD** (olmesartan 0.0054091 & Amlodipine 0.0016675**) and LOQ** (olmesartan 0.0163916 Amlodipine 0.0050529), **Accuracy** (% Recovery more than 99.5 for both drugs), **Precision** (mean % Recovery more than 99.5 for both drugs), **Ruggedness and Robustness.** All validation parameter are under limits that suggested by ICH guideline.

CONCLUSION

We can conclude that the HPLC methods for quantitative estimation of pharmaceuticals are fast, less time consuming, reproducible and highly sensitive even microgram of compound

can be measured. Performing a through method validation can be a tedious process, but the quality of data generated with the method is directly linked to the quality of this process. Time constraints often do not allow for sufficient method validations. Many researchers have experienced the consequences of invalid methods and realized that the amount of time and resources required to solve problems discovered later exceeds what would have been expended initially if the validation studies had been performed properly.

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