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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF ALISKIREN AND AMLODIPINE IN TABLET DOSAGE FORM

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ABSTRACT

A simple, specific, precise, accurate, rapid and isocratic reverse phase high performance liquid chromatography (RP-HPLC) method was developed and validated for simultaneous estimation of Aliskiren and Amlodipine in tablet dosage form. RP-HPLC method development, the estimation was carried out by using the Kromasil, ODS 3V (250 x 4.6mm, 5µm) C_{18} column with 5µm particle size. Injection volume of 20µl is injected and eluted with the mobile phase Phosphate buffer: Acetonitrile in the ratio of 60: 40, which is pumped at a flow rate of 1.0 ml/min. Detection, was carried out at 237 nm using photodiode Array (PDA) detector. The retention time for Aliskiren and Amlodipine was respectively 3.9, 5.0mins. The method was precisely applied to the tablet formulation and the results obtained were accurate and reproducible. The method was successfully validated in terms of linearity, precision, accuracy& robustness, LOD, LOQ as per ICH guidelines. Using the optimized chromatographic conditions, chromatograms of Aliskiren and amlodipine were recorded. Calibration curves were obtained by using peak area vs. concentration. Aliskiren shows linearity in the range of 25-150µg/ml and Amlodipine shows linearity in the range of 2.5-15µg/ml correlation co-efficients was found to be 0.9979 and 0.9973. The accuracy studies were shown as % recovery for Aliskiren and Amlodipine at 50%, 100% and 150%. The limit of % recovered shown is in the range of 98-102% and the results obtained were found to be within the limits. Hence the method was found to be accurate. For Intra-Day & Inter-day precision studies of Aliskiren and Amlodipine was performed. %RSD was determined from the peak areas and was found to be not more than 2%. The proposed method is simple, accurate and rapid. Limit of detection (LOD) and Limit of quantification (LOQ) were estimated from the signal-to-noise ratio. Limit of detection of 1.37741µg/ml & 4.17396µg/ml & limit of quantification of 0.73967µg/ml and 2.24142µg/ml for Aliskiren & Amlodipine respectively. For robustness studies the chromatograms were recorded for standard solutions of Aliskiren and Amlodipine by changing flow rate. Robustness studies reveal that the method was reliable.

KEY WORDS

Aliskiren and Amlodipine, RP-HPLC

INTRODUCTION

Aliskiren is an Anti-hypertensive agent. Chemically Aliskiren, 2(2S, 4S, 5S, 7S)-5-amino-N-(2-carbamoyl-2, 2-dimethylethyl)-4-hydroxy-7- {[4-methoxy- 3- (3-methoxypropoxy) phenyl] methyl}-8-methyl-2-(propan-2-yl) nonanamide. Renin is secreted by the kidney in response to decreases in blood volume and renal perfusion. Renin cleaves angiotensinogen to form the inactive decapeptide angiotensin I (Ang I). Ang I is converted to the active octapeptide angiotensin II (Ang II) by angiotensin-converting enzyme (ACE) and non-ACE pathways Aliskiren is a direct renin inhibitor,

decreasing plasma renin activity (PRA) and inhibiting the conversion of angiotensinogen to Angiotensin- I. Aliskiren is used in the treatment of hypertension. [1, 2].

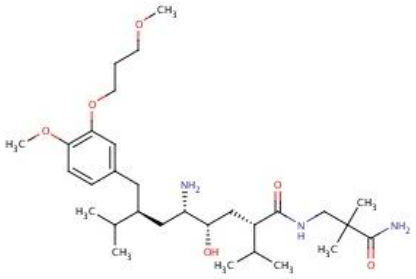

Figure 1: Chemical Structure of Aliskiren

Amlodipine is a calcium channel blocker, Anti-hypertensive agent. Amlodipine, chemically it is found 3-ethyl 5-methyl 2 -[(2-aminoethoxy) methyl] – 4 -(2 -chlorophenyl) -6-methyl -1, 4-dihydropyridine-3, 5-dicarboxylate. Amlodipine is a long-acting 1, 4-dihydropyridine calcium channel blocker. It acts primarily on vascular smooth muscle cells by stabilizing voltage-gated L-type calcium channels in their inactive conformation. Amlodipine decreases arterial smooth muscle contractility and subsequent vasoconstriction by inhibiting the influx of calcium ions through L-type calcium channels. Amlodipine is a long-acting CCB that may be used to treat mild to moderate essential hypertension and chronic stable angina. [3, 4]

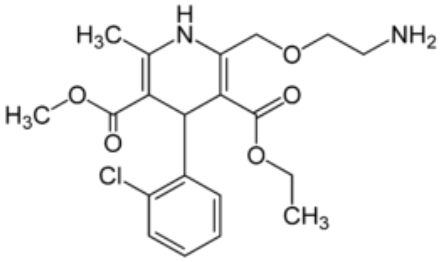

Figure 2: Chemical Structure of Amlodipine

Literature survey shows that various methods have been reported for estimation of either Aliskiren and Amlodipine alone or in combination with other drugs in pharmaceutical dosage forms or individually in biological fluids by UV- spectrophotometer, RP-HPLC, LC-MS. Three Spectrophotometric methods were established for the estimation of Aliskiren and Amlodipine in the combined dosage form. There were no HPLC methods established for the estimation of Aliskiren and Amlodipine in the novel formulation at the time of the present work. The aim of the present work is to develop an analytical method for the combined fixed dose tablet formulation of Aliskiren & Amlodipine, which is novel to the market. The work of interest is Aliskiren and Amlodipine in the combined dosage form. Validation of the method was done in accordance with ICH guidelines for the assay of active ingredients. Thus validated method can be recommended for the routine laboratory analysis.

MATERIALS & METHODS

Materials:

Analytically pure samples of Aliskiren (ALK) and Amlodipine (AML) were procured as gift samples from Dr. Reddy's Laboratories, (Hyderabad, India).TEKAMLO (Aliskiren-150mg and Amlodipine 10mg) tablets manufactured by NOVARTIS., USA were procured from a local pharmacy. The solvents for the experiment were selected based on the solubility test results of both the drugs. The solubility tests were performed using the common solvents like water, methanol (Sd Fine), Acetonitrile (Merck). The analytical reagent grade potassium di hydrogen phosphate (Qualikems fine chemicals pvt.ltd, vadodara) and orthophosphoric acid was used to

prepare the mobile phase which is filtered through a nylon 0.45µm membrane filter paper.

CHROMATOGRAPHIC CONDITIONS:

Method was developed Using a Shimadzu UFLC-20AD chromatographic system (Japan), equipped with isocratic pump, and with SPD-M20A diode array detector attached with data recorder and integrator (LC solution) software.HPLC with PDA detector (Waters); HPLC column using is Kromasil ODS 3V (250 x 4.6mm, 5µm); Mobile phase filtration unit was Ultipor Nylon membrane (Pall Life sciences, Mumbai, India).

PREPARATON OF STANDARD STOCK SOLUTION:

Accurately Weighed and transferred 50mg of Aliskiren and 10mg of Amlodipine working Standards into a 10 ml clean dry volumetric flask, add 3ml of diluent, sonicated for 5 minutes and make up to the final volume with diluents to get a concentration of 5000 µg/ml of Aliskiren and 1000 µg/ml of Amlodipine (Stock Solution). From stock solution Aliskiren 0.3 ml and Amlodipine 0.1ml was taken in 10 ml volumetric flask and volume was made up to the mark with diluent diluents to get a concentration of 150µg/ml of Aliskiren and 10µg/ml of Amlodipine (Standard Solution).

PREPARATION OF SAMPLE SOLUTION:

Twenty tablets of TEKAMLO containing ALK and AML 150mg: 10mg respectively were weighed and crushed to fine powder and calculated the average weight of each tablet. Powder equivalent to 150mg Aliskiren and 10mg of Amlodipine was weighed and transferred into a 250 mL volumetric flask, dissolved in 60 ml of diluents and sonicated for 25 min and filtered through 0.45µ filter. From the filterate 0.5 ml was pipeted and transferred into a 10ml volumetric flask and the solution was made up to the volume with diluents.

METHOD VALIDATION: The developed HPLC method for simultaneous determination of Aliskiren and Amlodipine formulation was validated as per ICH guidelines.

ASSAY OF FORMULATION:

Preparation of Standard stock solution:

Accurately Weighed and transferred 50mg of Aliskiren and 10mg of Amlodipine working Standards into a 10 ml clean dry volumetric flask, add 3ml of diluent , sonicated for 5 minutes and make up to the final volume with diluents& filtered through 0.45µ membrane filter to get a stock solution containing 5000µg/ml and 1000µg/ml of Aliskiren & Amlodipine respectively.

Preparation of Standard solution:

From stock solution Aliskiren 0.3 ml and Amlodipine 0.1ml was taken in 10 ml volumetric flask and volume was made up to the mark with diluents, to get a standard solution containing 150µg/ml Aliskiren and 10µg/ml of Amlodipine respectively (Standard Solution).

Sample Preparation:

Twenty tablets were weighed and crushed to fine powder. The tablet powder equivalent to 150 mg of Aliskiren , 10mg of Amlodipine was transferred to a 250 ml volumetric flask and dissolved in mobile phase and the content was made up to mark with mobile phase, Then the sample solution kept in sonicater for 15min, then filtered the solution through 0.45µm filter paper . Final concentration of Aliskiren, Amlodipine of 150µg/ml, 10µg/ml are made by suitable dilutions**.** From the filtered solution 0.5ml was pipeted out into a 10 ml volumetric flask and made up to 10ml with diluents, to get concentration containing 50µg/ml.

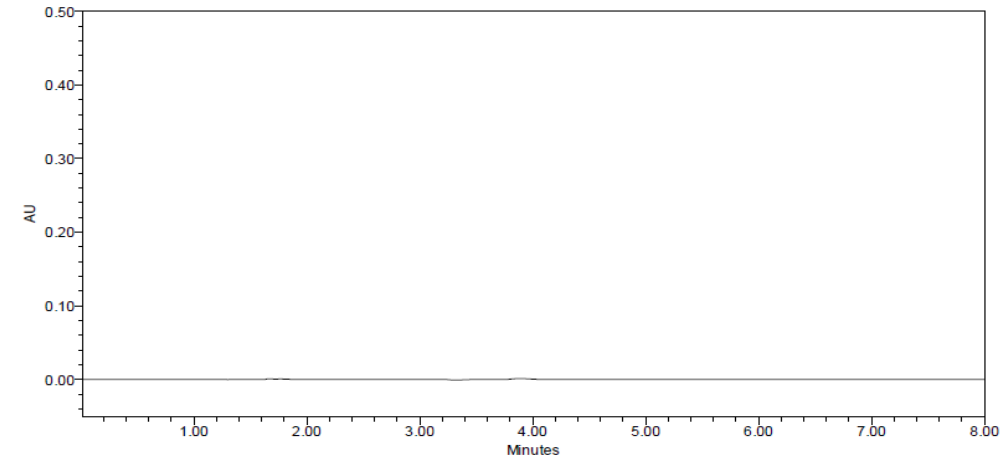

Figure 3: Chromatogram of Blank

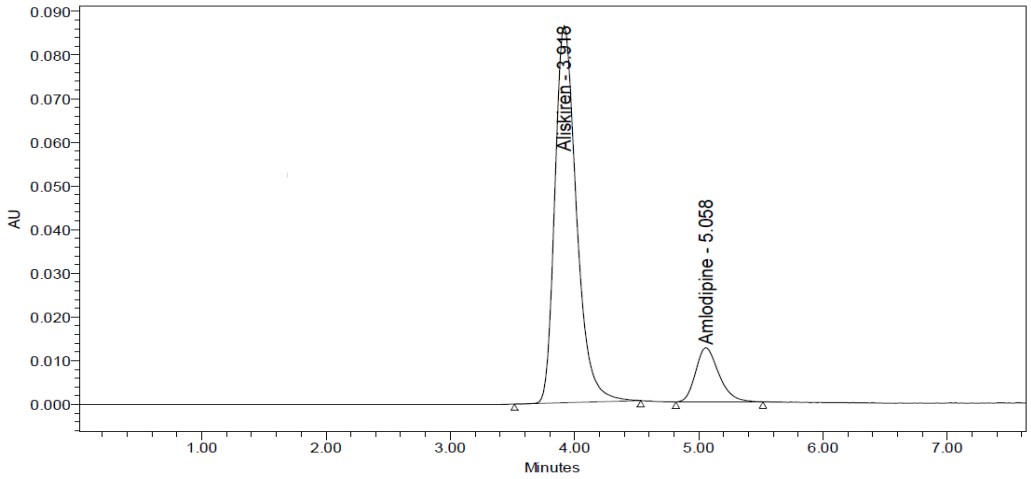

Figure 4: Chromatogram of Standard

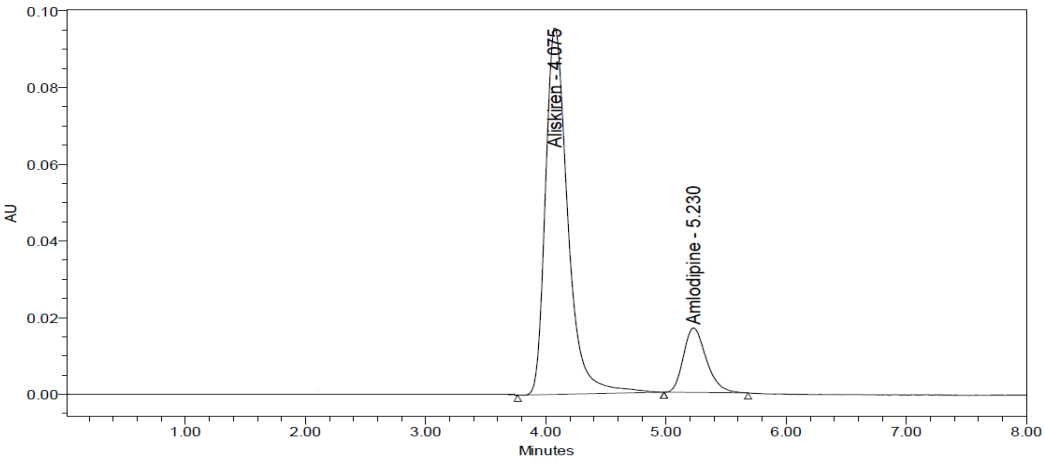

Figure 5: Chromatogram of Test

Table 1: Peak results of Standard & Test Chromatograms for Assay

Parameter	Standard		Test	
	Aliskiren	Amlodipine	Aliskiren	Amlodipine
Retention time	3.918	5.058	4.075	5.230
Peak Area	1055681	160471	1050454	160707
USP Plate Count	2606	3756	3101	4094
Tailing Factor	1.3	1.2	1.3	1.2

The percentage assay is given by the following formula:

$$\%\ Assay = \frac{At}{As} \times \frac{Ws}{Ds} \times \frac{Dt}{Wt} \times \frac{P}{100} \times \frac{avg.weight}{label\ claim} \times 100$$

Where,

At = average area counts of sample preparation.
As = average area counts of standard preparation.
Ws = Weight of working standard taken in mg.
Wt = Weight of sample taken in mg.
Dt = sample dilution
Ds = standard dilution
P = Percentage purity of working standard

$$\text{Amount found (mg)} = \frac{\text{\% of Drug}}{100} \text{ x label claim}$$

The % assays of Aliskiren and Amlodipine were found to be 99.4% and 100.5% respectively. Thus, % Assay results were found to be within the limits i.e., 98-102% for both the drugs. Hence the developed method can be routinely used for the simultaneous estimation of Aliskiren and Amlodipine in the marketed formulations.

Method validation was performed as per the ICH guidelines. The developed method was validated for the following parameters.

A. System suitability
B. Linearity
C. Specificity
D. Precision
E. Accuracy
F. LOD & LOQ
G. Robustness

SYSTEM SUITABILITY TEST (SST)

Six replicate injections of standard solution were injected and the chromatograms were recorded. The system was suitable for analysis if the % relative standard deviation (%RSD) of area counts in five replicate injections should be not more than 2.0%. USP tailing factor for Aliskiren and Amlodipine peak should be not more than 2.0. USP resolution factor between the peaks corresponding to Aliskiren and Amlodipine should be more than 2.0.

LINEARITY:

The linearity of an analytical method 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.

Table 2: Preparation of working standard solutions for Linearity

Working standard solutions (Level in %)	Stock solution taken in (ml)	Stock Solution taken in (ml)	Diluted to volume (ml) with diluents	Concentration of Aliskiren (µg/ml)	Concentration of Amlodipine (µg/ml)
25%	0.075	0.025	10	25	2.5
50%	0.15	0.05	10	50	5
75%	0.225	0.75	10	75	7.5
100%	0.3	0.1	10	100	10
125%	0.375	0.125	10	125	12.5
150%	0.45	0.15	10	150	15

Where, σ = the standard deviation of the response,
S = slope of the calibration curve.

SPECIFICITY:

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Blank Interference:

A study to establish the interference of blank was conducted. Volume of 20µl of diluent was injected twice into the HPLC system & the chromatogram was recorded.

PRECISION:

The precision of analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of series of measurement.

System Precision:

System precision was established to ensure that the optimized analytical method is precise. System precision was performed by injecting six replicate injections of standard solution at 100% concentration and the chromatograms were reviewed for the %RSD of peak areas. % RSD of the assay value for six determinations should not be more than 2.0%.

Method precision:

Method precision also called as repeatability/Intra-day precision indicates whether a method gives consistent results for a single batch. Method precision was demonstrated by preparing six test solutions at 100% concentration as per the test procedure & recording the chromatograms of six test solutions. The % RSD of peak areas of six samples was calculated. The method precision was performed on Aliskiren and Amlodipine formulation. The % RSD of the assay value for six determinations should not be more than 2.0%.

ACCURACY:

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value.

The accuracy of an analytical method should be established across its range. Accuracy is performed in three different levels for Aliskiren and Amlodipine at 50%, 100% and 150%. Samples analyzed at each level in triplicate. From the results, % recovery was calculated. Average % recovery at each spike level not less than 98.0 and not more than 102.0.

LIMIT OF DETECTION & QUANTIFICATION:

In the present study, the LOD and LOQ were calculated according to the standard deviation of the response and the slope of the calibration curve i.e., 3.3σ / S and 10σ / S criterions, respectively; where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve.

ROBUSTNESS:

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and

provides an indication of its reliability during normal usage.

Robustness was done by changing the mobile phase (±2ml), flow rate (±1%), changing the wavelength (±5nm). All the system suitability parameters must be met as per the method.

Effect of variation in flow rate:

A study was conducted to determine the effect of variation in flow rate. Standard and Test solutions of 100% concentration was prepared & injected into the HPLC system by keeping flow rates 0.9 ml/min & 1.1 ml/min. The effect of variation of flow rate was evaluated.

Effect of variation in mobile phase composition: A study was conducted to determine the effect of variation in mobile phase ratio by changing the ratio of organic solvent i.e., Buffer: Acetonitrile by ± 2ml. Standard & test solutions of 100% concentration were prepared and injected into the HPLC system and the chromatograms were recorded. The retention times, tailing factors & %RSD values were calculated.

RESULTS AND DISCUSSION

The present work was performed successfully by scheduling the entire work into steps and performing each & every step in the best possible way by sorting out and rectifying the problems & errors.

Solubility of Aliskiren & Amlodipine was performed in various organic & inorganic solvents. Combination of Aliskiren & Amlodipine was found to be insoluble in 0.1N NaOH, soluble in Water, methanol, ethanol, acetonitrile.

From the overlain spectrum, the Isoabsorptive point was found to be 235nm & also it was observed that both the drugs show a near maximum absorbance in the range of 230-250nm. Since the formulation TEKAMLO contains a very low quantity (10mg) of Amlodipine, it is a need to select a wavelength at which Amlodipine shows the maximum absorbance & Aliskiren shows a near maximum absorbance. Hence, mixed standard solution containing both the drugs in same was chromatographed at different detection wavelengths ranging from 230-250nm. At, 237nm Amlodipine showed the maximum absorbance with Aspirin showing a significant near maximum absorbance. Hence, 237nm was selected as the detection wavelength.

Optimization of the Chromatographic conditions was performed by running several trials to obtain retention times, peak symmetry, plate count, resolution within limits & possibly the best. In RP-HPLC method, the conditions were optimized to obtain an adequate separation of eluted compounds. Initially, various mobile phase compositions were tried, to separate title ingredients. Mobile phase and flow rate selection was based on peak parameters (height, tailing, theoretical plates, capacity or symmetry factor), run time and resolution. The mobile phase containing mixture of potassium dihydrogen orthophosphate buffer solution: Acetonitrile (60:40v/v, pH 3) with a flow rate of 1.0 ml/min is quite robust. The optimum wavelength for detection was 237 nm at which better detector response for both the drugs was obtained. The retention times for Aliskiren and Amlodipine was found to be 3.918 ± 0.005 min and 5.058± 0.009 min, respectively.

Assay was performed using the optimized method was for the tablet formulation TEKAMLO. % Assay values for ALK & AML were 99.4% & 100.5% respectively i.e., found to be within the limits of 98-102%. Hence, it can be concluded that the method showed no interference due to the excipients & hence, the optimized method can be used for the routine analysis of the marketed formulations of the drugs of interest.

From the **system suitability studies**, it was observed that %RSD of peak areas was found to be 0.9 for Aliskiren & 0.5 for Amlodipine. Theoretical plate count was found to be 2606 & 3756 for Aliskiren & Amlodipine respectively. USP tailing factor was found to be 1.33 & 1.26 for Aliskiren & Amlodipine respectively. All the performance parameters were within the limits.

From the **linearity** data, it was observed that the method was linear over the concentration range of 25 to 150µg/ml & 2.5 to 15µg/ml for Aliskiren & Amlodipine respectively. Correlation coefficient was found to be 0.9979 & 0.9973 for Aliskiren & Amlodipine respectively.

The **precision** of the method was determined at various levels in the name of system precision, method precision and intermediate precision. The

peak areas of the standard chromatograms and the %assay values of the test chromatograms were expressed in terms of %RSD which was within the acceptable limits of 2%, which indicates good precision. The % recovery of Aliskiren & Amlodipine at each level was within the limits of 98% and 102%. Hence, accuracy was established for the present work and the method was said to be accurate.

Robustness of the proposed method was determined by varying various parameters, the %RSD reported was found to be less than 2 %.

As the system suitability parameters for the standard and test chromatograms of Aliskiren & Amlodipine were within limits for variation in flow rate (±0.1ml) and mobile phase comoposition, the allowable variation in flow rate and organic solvent ratio in mobile phase composition should be 1±0.1ml/min and 60±2ml respectively. Also, it can be concluded that the method was **robust**.

The **lowest** possible concentration of Aliskiren that can be **detected and quantified** by the present method was found to be 1.37741µg/ml and 4.17396µg/ml respectively and that of Amlodipine was found to be 0.73967µg/ml and 2.24142µg/ml respectively.

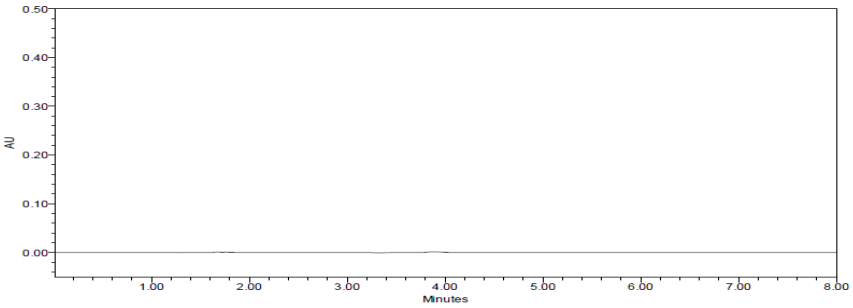

Figure 6: Chromatogram of Blank for System suitability

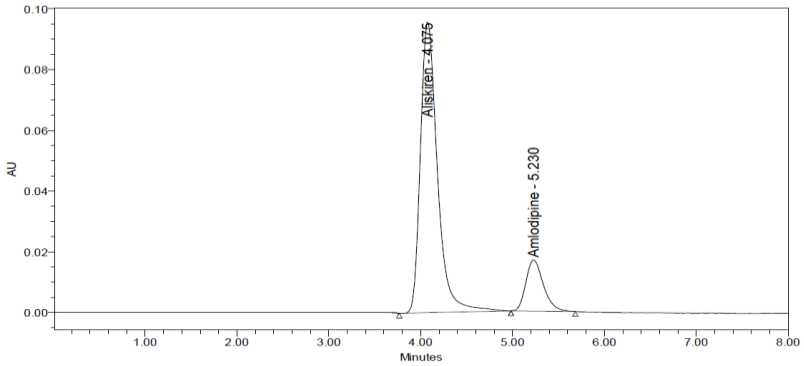

Figure 7: A Representative Chromatogram for System suitability

Table 3: Results of System suitability Test for Aliskiren

Injection	Retention time (t_R)	Peak Area	Plate count	Tailing factor
1	3.884	1055681	2606	1.33
2	3.888	1055535	2591	1.34
3	3.904	1061247	2538	1.34
4	3.931	1038510	2662	1.33
5	3.971	1057476	2549	1.33
6	3.998	1066610	2644	1.35
Mean	-	1055843	-	-
SD	-	9469.7	-	-
% RSD	**-**	0.9	-	-

Table 4: Results of System suitability Test for Amlodipine

Injection	R_t (min)	Area	Plate Count	Tailing Factor
1	5.003	160471	3756	1.26
2	5.007	161936	3651	1.30
3	5.029	161727	3639	1.25
4	5.064	162161	3624	1.30
5	5.115	162422	3590	1.32
6	5.146	160688	3675	1.25
Mean	-	**161567**	-	-
%RSD	-	**0.5**	-	-

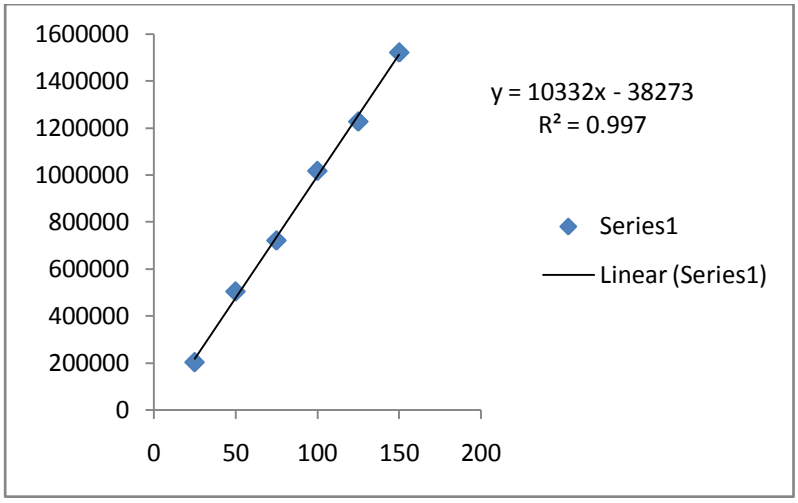

Figure 8: Linearity graph of Aliskiren

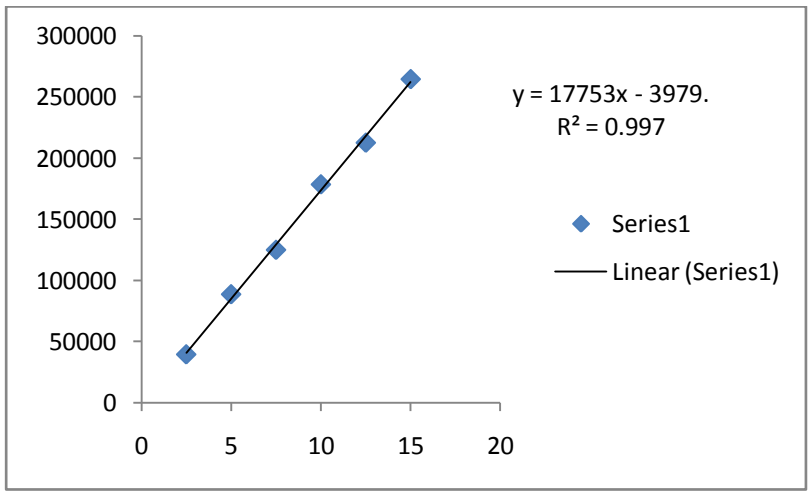

Figure 9: Linearity graph of Amlodipine

Table 5: Method Precision or Intra Day Precision of Aliskiren

S.No	Concentration (µg/ml)	Retention time(Rt)	Peak Area	%Assay
1	20	4.053	1054687	99.79
2	20	4.087	1055987	99.90
3	20	4.112	1059234	100.22
4	20	4.128	1053557	99.68
5	20	4.131	1057946	100.10
6	20	4.150	1056167	99.93
AVG		_	1056246	99.94
%RSD		_	**0.2**	**0.20**

Table 6: Method Precision of Amlodipine

S.No	Concentration (µg/ml)	Retention time(Rt)	Peak Area	%Assay
1	10	5.221	162068	100.21
2	10	5.271	162369	100.40
3	10	5.295	162394	100.41
4	10	5.314	162635	100.56
5	10	5.322	160820	99.44
6	10	5.337	162051	100.20
AVG		_	162056	100.202
%RSD		_	**0.4**	**0.398**

Table 7: Intermediate Precision data for Aliskiren and Amlodipine

	Intermediate Precision			
S.No.	**Day 1 Aliskiren Retention time(Rt)**	**Peak Areas**	**Day 1 Amlodipine Retention time(Rt)**	**Peak Areas**
1	4.142	1029388	5.279	170328
2	4.164	1015957	5.313	170684
3	4.197	1035949	5.364	173609
4	4.202	1018770	5.370	172086
5	4.211	1032187	5.372	168628
6	4.219	1032120	5.376	170801
Avg		1027395		171023
SD		8094.9		1685.3
%RSD		0.8		1.0

Table 8: Intermediate Precision data for Aliskiren and Amlodipine

	Intermediate Precision			
S.No.	Day 2 Aliskiren		Day 2 Amlodipine	
	Retention time(Rt)	Peak Areas	Retention time(Rt)	Peak Areas
1	4.132	1022388	5.260	170228
2	4.146	1015847	5.309	170694
3	4.157	1035959	5.344	173606
4	4.201	1016780	5.362	172084
5	4.219	1030187	5.372	168626
6	4.221	1032220	5.378	170808
Avg		1027245		171025
SD		8083.9		1673.3
%RSD		0.7		2.0

Table 9: Intermediate Precision data for Aliskiren and amlodipine

	Intermediate Precision			
S.No.	Day to Day Aliskiren		Day to Day Amlodipine	
	Peak Area	%Assay	Peak Area	%Assay
1	1029388	97.39	170328	105.31
2	1015957	96.12	170684	105.53
3	1035949	98.01	173609	107.34
4	1018770	96.39	172086	106.4
5	1032187	97.66	168628	104.26
6	1032120	97.65	170801	105.60
Avg	1027395	97.20	171023	105.74
SD	8094.9	0.7653	1685.3	1.0425
%RSD	0.8	0.7	1.0	0.9

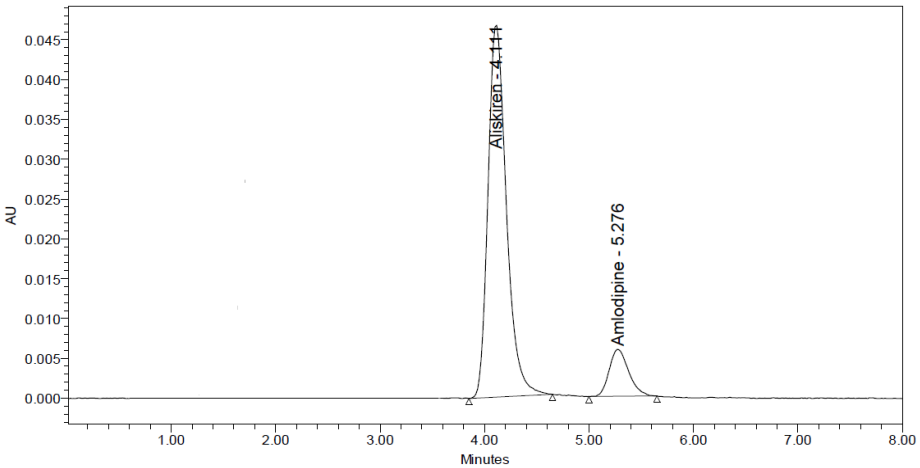

Figure 10: Chromatograms for Accuracy level -50%

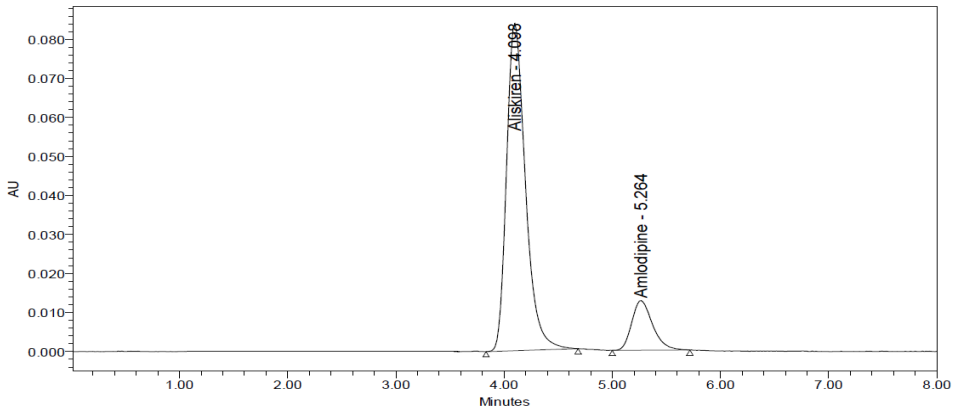

Figure 11: Chromatograms for Accuracy level -100%

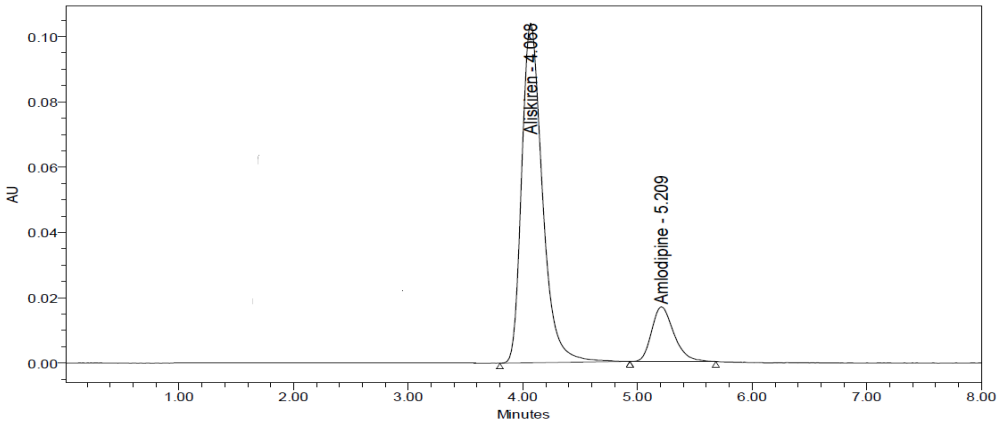

Figure 12: Chromatograms for Accuracy level -150%

Table 10: LOD and LOQ Data of Aliskiren and Amlodipine

ALISKIREN			AMLODIPINE		
Conc.(x) (µg/ml)	**Peak Areas (y)**	**Statistical Analysis**	**Conc.(x) (µg/ml)**	**Peak Areas (y)**	**Statistical Analysis**
2	26031		2	7388	
4	47099	S = 10332 c = 38273	4	10740	S = 17753 c = 3979.2
		LOD:1.37741µg/ml LOQ:4.17396µg/ml			LOD: 0.73967µg/ml LOQ: 2.24142µg/ml

Where,
S: Slope of respective calibration curve
c: y-intercept
σ: Standard deviation of y-intercepts

CONCLUSION

A simple, specific, precise, accurate, rapid and isocratic reverse phase high performance liquid chromatography (RP-HPLC) method was developed and validated for simultaneous estimation of Aliskiren and Amlodipine in tablet dosage form.

A simple, economic, accurate and precise HPLC method was successfully developed. The method was successfully validated in terms of linearity, precision, accuracy& robustness, LOD, LOQ as per ICH guidelines.

Using the optimized chromatographic conditions, chromatograms of Aliskiren and amlodipine were recorded. Calibration curves were obtained by using peak area vs. concentration. The accuracy studies were shown as % recovery for Aliskiren and Amlodipine at 50%, 100% and 150%. The limit of % recovered shown is in the range of 98-102% and the results obtained were found to be within the limits. Hence the method was found to be accurate. For Intra-Day & Inter-day precision studies of Aliskiren and Amlodipine was performed. %RSD was determined from the peak areas and was found to be not more than 2%. The proposed method is simple, accurate and rapid.

Limit of detection (LOD) and Limit of quantification (LOQ) were estimated from the signal-to-noise ratio. Limit of detection of 1.37741µg/ml & 4.17396µg/ml & limit of quantification of 0.73967µg/ml and 2.24142µg/ml for Aliskiren & Amlodipine respectively. For robustness studies the chromatograms were recorded for standard solutions of Aliskiren and Amlodipine by changing flow rate. Robustness studies reveal that the method was reliable.

Hence the proposed method was found to be rapid, accurate, precise, robust and economical. The mobile phase is simple to prepare and economical. This method is also having an advantage of short retention time. The proposed method was a good approach for obtaining reliable results & found to be suitable for the routine analysis and quality control of pharmaceutical preparations containing these drugs either individually or in combination.

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