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Validated RP-HPLC Method for the Simultaneous Determination of Aliskiren, Hydrochlorothiazide and Amlodipine besylate in Pharmaceutical Formulations

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Abstract: A reverse phase high performance liquid chromatographic method has been developed for the simultaneous estimation of Aliskiren hemifumarate (ALN), Amlodipine besylate (AMB) and hydrochlorothiazide (HCT) in pharmaceutical formulation using RP-C_{18} Column. The mobile phase (Acetonitrile: methanol: phosphate buffer adjusted to pH 3.0± 1 with orthophosphoric acid) was pumped at a flow rate of 1.0 mL/ min in the ratio of 20:50:30% v/v and the eluent was monitored at 239 nm. Linearity was obtained in the concentration range of 5 –30 µg/ mL for Amlodipine besylate, 75-450 µg/ mL for Aliskiren and 12.5 -75 µg/ mL for hydrochlorothiazide. The method was statistically validated and RSD was found to be less than 2% indicating high degree of accuracy and precision of the proposed HPLC method. Due to its simplicity, rapidness, high precision and accuracy, the proposed HPLC method can be applied for determining Aliskiren, Amlodipine and hydrochlorothiazide in bulk and in pharmaceutical dosage form.
Key words: RP-HPLC method, Aliskiren, Amlodipine besylate, Hydrochlorothiazide Development and validation, Tablets.

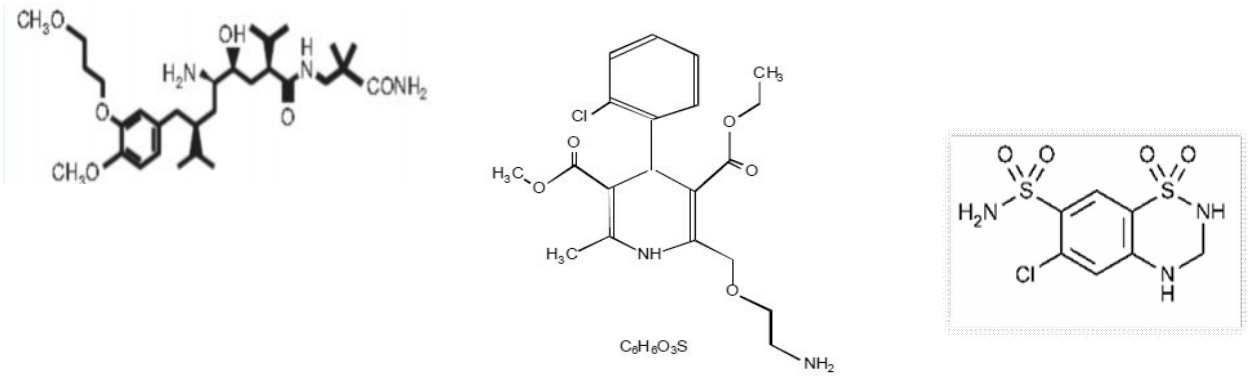
Introduction:

Aliskiren hemifumarate (ALN)[1-3], (2S, 4S, 5S, 7S)-N-(2-carbamoyl-2-methylpropyl)-5-amino-4-hydroxy-[2,7]-diisopropyl-8-[4-methoxy-3-(3-methoxypropoxy)phenyl]-octanamide hemifumarate is a Rennin inhibitor, Antihypertensive Agent**.** Various analytical methods have been reported for the assay of ALN alone or in combination with other anti - hypertensive agents in pharmaceutical formulations.

Amlodipine besylate (AMB)[1-3] 2-[(2 - amino ethoxy) - methyl] - 4 - (2 – chloro phenyl) -1, 4 -dihydro - 6 - methyl - 3, 5 - pyridine dicarboxylic acid 3 - ethyl - 5 - methyl ester, benzene sulfonate, is a potent dihydro calcium channel blocker.

Hydrochlorothiazide (HCT)[1-3] 6 - chloro - 3, 4 - dihydro - 7 - sulfamoyl - 2H - 1, 2, 4 - benzothia - diazine - 1, 1 – dioxide, is a thiazide diuretic. It increases sodium and chloride excretion in distilled convoluted tubule. Many analytical methods for HCT alone and in combination with other drugs by stability indicating method, RP- HPLC methods, spectro photometric methods and in plasma.

All the three drugs are official in USP[4]. Amlodipine besylate and Hydrochlorothiazide are official in IP[5]. The chemical structures of ALN, AMB, and HCT are shown in figure 1.



(A) (B) (C)

Fig 1. The chemical structures of Aliskiren hemifumarate (A) Amlodipine besylate (B), Hydrochlorothiazide(C)

Literature survey revealed that there are several methods were reported for the estimation of ALN, AMB and HCT individually as well as in combination with some other drugs. As no method is available for their simultaneous determination , however, it is essential to develop a suitable analytical method for simultaneous estimation of ALN, AMB and HCT in bulk and in pharmaceutical preparations, because HPLC methods have been widely used for routine quality control assessment of drugs, because of their accuracy, repeatability, selectivity, sensitivity and specificity. We have developed a simple, precise, accurate and specific RP-HPLC method for the simultaneous determination of ALN, AMB and HCT in bulk and in pharmaceutical dosage forms. Because analytical methods must be validated before use by the pharmaceutical industry, the proposed HPLC- UV detection method was validated in accordance with International conference in Harmonization (ICH) guidelines[6-8] by assessing its selectivity, linearity, accuracy, precision, limit of detection and limit of quantification.

Experimental:

Instrumentation:

Analysis was performed with a Waters 515 chromatograph equipped with Hiber@ Lichrosphere ® C_{18} (250 mm × 4.6 mm i.d., 5µm) 510 Pumps and waters-2487- UV-Visible detector. Samples were injected into the system through a Rheodyne 7725 injection valve via a 50 µL loop. The output signal was monitored and integrated by Empower-2 software. Lichrosphere® C_{18} (250 mm × 4.6 mm i.d., 5µm) was used for this method. Solubility of the compound was enhanced by sonication on an ultra sonicator (PCI Analytics PCI81). All the weighings in the experiments were done with Shimadzu balance (model AX200). PVDF membrane filters were purchased from Merck Millipore.

Reagents and chemicals

Pharmaceutically pure samples of Aliskirn hemifumarate (ALN) was obtained as a gift samples from Morpen laboratories, New Delhi. AMB and HCT were obtained as a gift samples from Dr.Reddy's, Hyderabad. A combination of ALN (300 mg), AMB (10 mg) and HCT (25 mg) in tablet formulation (**Amturnide**) was procured from U.S market (Novartis pharmaceuticals, Mumbai). HPLC grade methanol, Acetonitrile, and water and potassium di hydrogen ortho phosphate (AR grade) were obtained from Qualigens India Pvt. Limited, Mumbai, India.

Preparation of buffer

About 2.72 g of Potassium dihydrogenphosphate was transferred in to a beaker containing 1000 mL of water and mixed .The pH of the solution was adjusted to 3.0 ± 0.02 with dilute ortho phosphoric acid. The solution was then filtered through a 0.45µ membrane filter and sonicated.

Preparation of diluent

A mixture of the Acetonitrile: Methanol: Phosphate buffer and methanol in the ratio of 20:50:30 v/v was used as the diluent.

Preparation of mobile phase

The above buffer (pH 3.0) was mixed thoroughly with acetonitrile and methanol in the ratio of 20:50:30 v/v. This solution was used as the mobile phase.

Preparation of mixed working standard solution of Aliskiren hemifumarate, Amlodipine basylate and Hydrochlorothiazide

25 mg , 5 mg and 12.5 mg of Aliskiren, Amlodipine and Hydrochlorothiazide was accurately weighed and transferred the working standards into a 10 mL, 25 ml and 25 mL clean dry volumetric flasks, added 6ml and 15 mL of diluent, sonicated for 30 minutes and made up to the final volume with diluent. From the above stock solution, 1.2 mL of Aliskiren, 0.5 ml of Amlodipine and Hydrochlorothiazide solution was pipetted out in to a 10 mL volumetric flask and then made with diluent and thus we have (300µg/mL Aliskiren, 10µg/mL Amlodipine and 25µg/mL Hydrochlorothiazide).

Optimization of the Method

The Mixture of acetonitrile: methanol: phosphate buffer (adjusted to pH 3.0 ± 1 with ortho phosphoric acid) in the ratio of 20:50:30 v/v was used as the mobile phase and pumped at a flow rate of 1 mL/ min. The detector wavelength was set at 239 nm. The injection volume was 10 µL. The separation was achieved at 25°C. Prior to injection of the drug solution, the column was equilibrated for at least 25 min by pumping the mobile phase through it. Typical chromatograms of the blank solution and mixed working standard solution of the combination of the drugs are shown in Fig. 2 and 3 respectively.

Chromatographic conditions

Column	:	Hiber@ Lichrosphere, (C_{18} 4.6×250mm 5.0µm)
Mobile phase ratio	:	ACN:Methanol: pH 3.0 buffer (20:50:30 (*v/v*)
Detection wavelength	:	239 nm
Flow rate	:	1.0 mL/min
Injection volume	:	10 µl
Column temperature	:	25^0C
Auto sampler temperature	:	25^0C
Run time	:	8 mins.
Retention time	:	2.6 mins for HCTZ, 5.8 mins for AMB and 6.4 mins for ALN

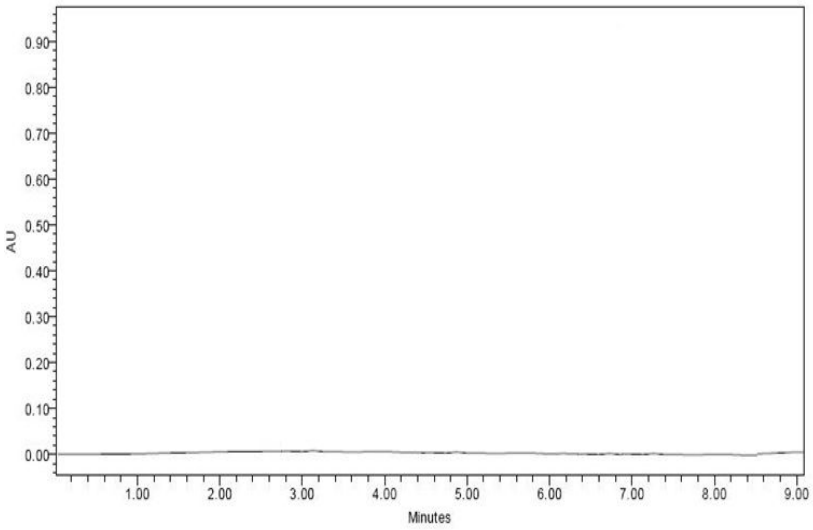


Fig 2 Chromatogram obtained from the analysis of blank solution

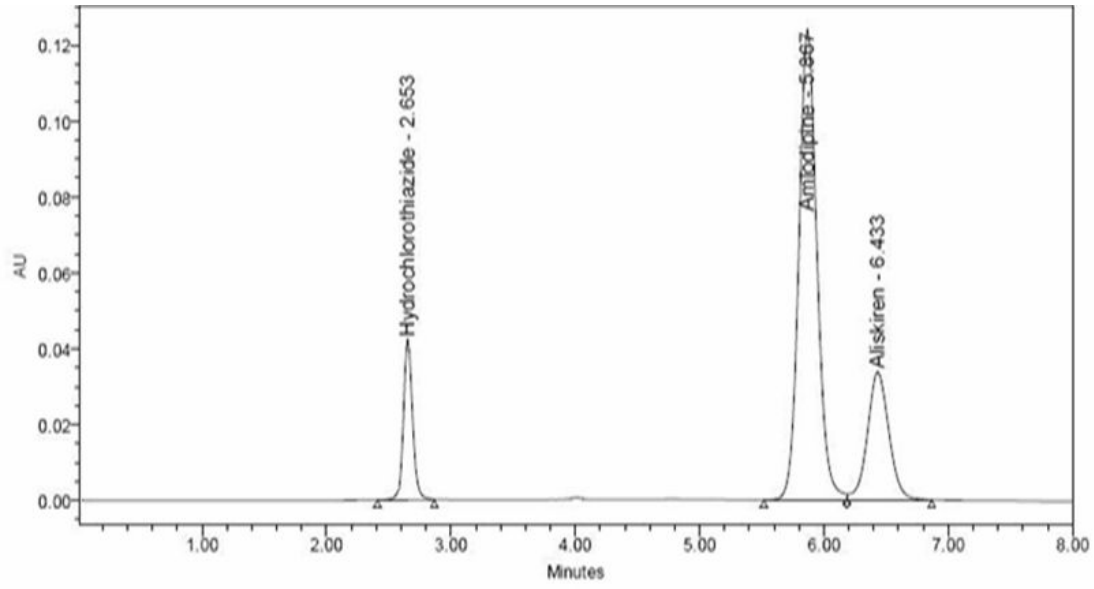


Fig 3 Typical Chromatogram of Aliskiren, amlodipine and Hydrochlorothiazide from the mixed working standard solution.

Method Validation

Linearity

The above standard solutions pippet out 0.3, 0.6, 0.9, 1.2, 1.5 and 1.8 mL of Aliskiren 0.125, 0.250, 0.375, 0.5, 0.625 and 0.75 ml of each Amlodipine and Hydrochlorothiazide were taken from stock solution of concentration 300 µg/mL Aliskiren, 10 µg/mL Amlodipine and 25 µg/mL Hydrochlorothiazide of Aliskiren, amlodipine and Hydrochlorothiazide and prepared at different concentration(75,150,225,300,375 and 450 µg/mL for aliskiren, 2.5,5.0,7.5,10.0,12.5 and 15.0 µg/mL for amlodipine and 6.25,12.5,18.5,25,31.25,37.5 µg/mL for hydrochlorothiazide) levels including the working concentration mentioned in the experimental condition were prepared. 10 µL of each concentration were injected into the HPLC system. The response was read at 239 nm and the corresponding chromatograms were recorded. From these chromatograms, the mean peak areas were calculated and linearity plots of concentration over the mean peak areas were constructed individually. Linearity data Aliskiren, amlodipine and Hydrochlorothiazide are given in the Table 1. Linearity plots for Aliskiren, amlodipine and Hydrochlorothiazide were depicted in Fig.4, 5 and 6 respectively.

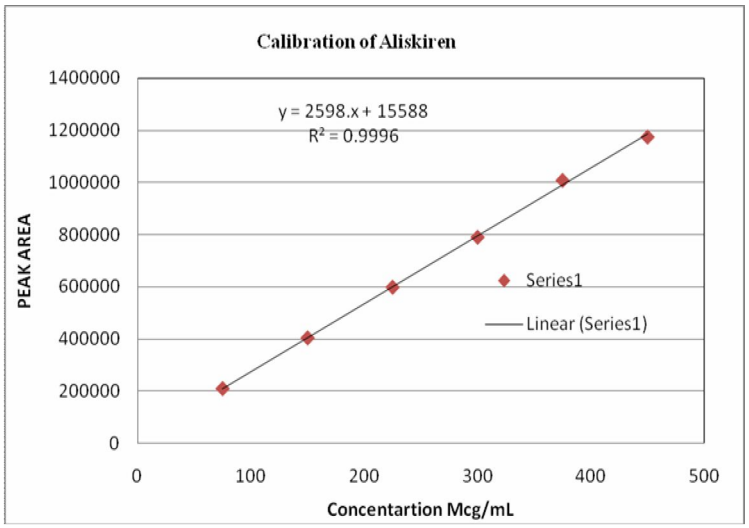


Fig.4.Calibration curve of Aliskiren hemifumarate

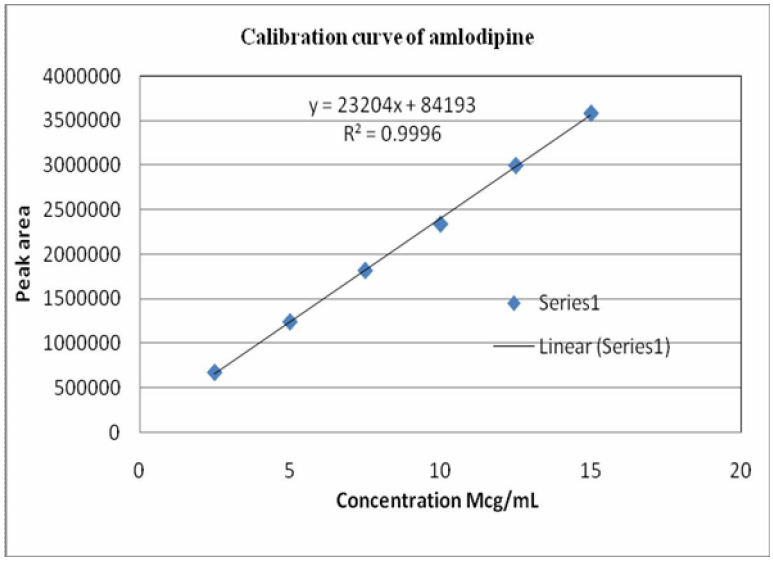


Fig 5. Calibration curve of Amlodipine besylate

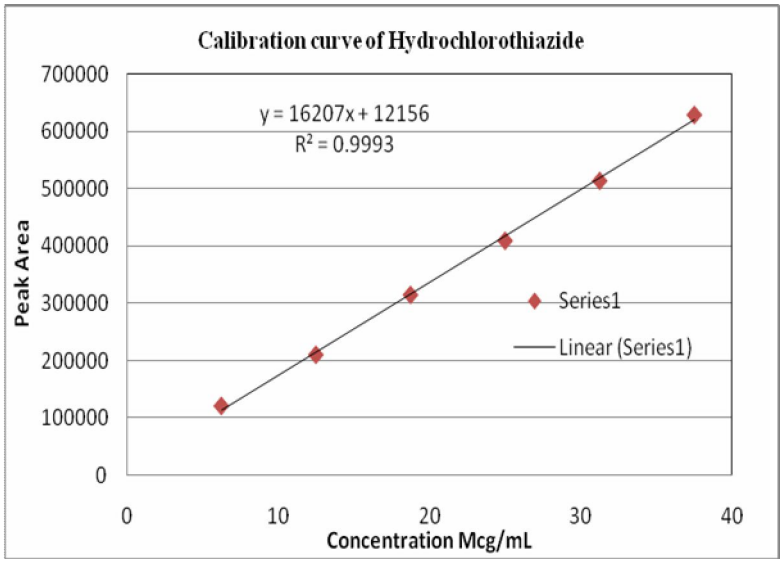


Fig 6. Calibration graphs of hydrochlorothiazide

Table 1.Linearity data Results

Level	ALN (µg/mL)	Peak area	AMB (µg/mL)	Peak area	HCTZ (µg/mL)	Peak area
Level-1	75	210414	2.5	681819	6.25	121274
Level-2	150	404644	5	1248232	12.5	210586
Level-3	225	598765	7.5	1824751	18.75	315195
Level-4	300	790344	10	2344538	25	409617
Level-5	375	1008211	12.5	3001376	31.25	514202
Level-6	450	1174362	15	3586741	37.5	629298
Slope		2598		2304		16207
Intercept		1558		84193		12156
Correlation Coefficient(r^2)		0.9996		0.9996		0.9993

Preparation of sample solution:

10 tablets were accurately weighed and calculated the average weight of each tablet then the weight equivalent to 10 tablets was transferred into a 100 mL volumetric flask, 50 mL of diluent added and sonicated for 25 min, further the volume made up with diluent and filtered. From the filtered solution 1mL was pipette out into a 1mL volumetric flask and made up to 10ml with diluent.

Precision

Repeatability and intermediate precision were assessed by analyzing 300µg/mL Aliskiren, 10 µg/mL Amlodipine and 25µg/mL Hydrochlorothiazide on the same day and consecutive days, respectively. The results of repeatability and intermediate precision studies are depicted in the Tables 2 and 3 respectively.

Table 2. Repeatability

S.NO	Aliskiren	Amlodipine	Hydrochlorothiazide
1.	792356	2356695	415965
2.	808407	2388258	423506
3.	807674	2380849	423437
4.	812332	2386385	424109
5.	814541	2394254	427009
6.	814457	2382451	423752
Average	808294.5	2381482	422963
SD	8339.42	13029.29	3682.322
RSD (%)	1.0317	0.5471	0.8706

Table 3.Intermediate precision

Drug	Sample No.	Labeled amount (mg/tab)	Amount found (mg)	Percentage obtained	Average (%)	S.D	% R.S.D.
ALN	1	300	299.85	99.42	99.32	0.8283	0.8339
	2	300	299.06	99.03			
	3	300	299.90	98.45			
	4	300	299.08	98.54			
	5	300	301.14	100.57			
	6	300	299.92	99.96			
AMB	1	10	9.9	99.87	99.40	0.6969	0.7011
	2	10	9.01	99.60			
	3	10	9.9	99.02			
	4	10	9.7	99.15			
	5	10	9.9	100.37			
	6	10	9.8	98.39			
HCT	1	25	24.49	98.72	99.16	0.3416	0.3445
	2	25	24.6	98.77			
	3	25	24.48	99.21			
	4	25	24.5	99.35			
	5	25	24.47	99.41			
	6	25	24.49	99.53			

Accuracy

The accuracy of the method was determined by spiking and analyzing in triplicate a known mixture of the drugs corresponding to 50 %, 100 % and 150 % levels of Aliskiren (50µg/mL, 100 µg/mL and 150 µg/mL), Amlodipine (5 µg/mL, 10 µg/mL, 15 µg/mL) and Hydrochlorothiazide (12.5 µg/mL, 25 µg/mL, 37.5 µg/mL). The percent recovery was calculated by noting the differences between the peak areas obtained for fortified and unfortified solutions. The results are incorporated in the Table 4.

Table 4. Recovery analysis of formulation (Amturnide)

% Level	Aliskiren			Amlodipine			Hydrochlorothiazide		
	Amount added (µg/mL)	Amount recovered (µg/mL	% Recovered	Amount added (µg/mL)	Amount recovered (µg/mL	% Recovered	Amount added (µg/mL)	Amount recovered (µg/mL	% Recovered
50	50	49.9	99.8	5	4.95	99.00	12.5	12.43	99.44
50	50	50.1	100.2	5	5.01	100.2	12.5	12.41	99.28
50	50	49.89	99.78	5	4.93	98.6	12.5	12.49	99.92
100	100	99.95	99.95	10	9.91	99.1	25	24.46	97.84
100	100	99.90	99.9	10	9.85	98.5	25	25.03	100.12
100	100	98.99	98.99	10	9.94	99.4	25	24.45	97.8
150	150	149.9	99.93	15	14.9	99.33	37.5	37.43	99.81
150	150	149.05	99.36	15	15.01	100.06	37.5	37.41	99.76
150	150	150.05	100.03	15	15.03	100.2	37.5	37.49	99.86
Average			99.77			99.37			99.31
S.D			0.3716			0.6534			0.8833
RSD			0.3742			0.06575			0.8894

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were calculated using residual standard deviation of the response and the slope of the regression line. The LOD and LOQ of Aliskiren, amlodipine and hydrochlorothiazide were found to be 0.1521 and 0.2305 µg/mL, 0.2132 and0.3015 µg/mL and 0.2517 and 0.2615 µg/mL respectively

Robustness study

The robustness of the method was determined as per ICH guidelines under a variety of conditions like change in flow rate and pH of buffer. The results obtained by deliberately variation in method parameters and data are summarized at Table 5, 6, 7.

Table 5 Robustness study- flow rate 0.9 mL/min

Drug	Retention time (min)	Tailing factor	Number of theoretical plates	Resolution
Aliskiren	7.52	1.5	4137	------
Amlodipine	6.84	1.8	3138	2.5
Hydrochlorothiazide	3.08	1.9	4739	2.5

Table 6 Robustness study- flow rate 1.1 mL/min

Drug	Retention time (min)	Tailing factor	Number of theoretical plates	Resolution
Aliskiren	8.35	1.3	5127	-------
Amlodipine	7.39	1.3	3738	4.9
Hydrochlorothiazide	2.75	1.5	7739	5.5

Table 7 Robustness study- More Organic composition

Drug	Retention time (min)	Tailing factor	Number of theoretical plates	Resolution
Aliskiren	6.55	1.1	6737	----
Amlodipine	6.04	1.3	4938	5.4
Hydrochlorothiazide	2.44	1.2	2939	6.5

Specificity and selectivity of the proposed method

Specificity is the extent to which the procedure applies to analyte(s) of interest and is checked by examining the formulation samples for any interfering peaks. The specificity of the method was evaluated with regard to interference due to presence of excipients. The excipients used in formulation did not interfere with the drug peaks and thus the method is specific. The HPLC chromatograms recorded for the drug matrix did not show any interfering peak within retention time ranges. Fig.2 and 3 shows the representative chromatograms obtained from the analysis of Aliskiren, Amlodipine and hydrochlorothiazide from blank solution, working standard solution and the formulation sample solution. The figures show that the selected drugs were clearly separated. Thus the proposed HPLC method is selective.

System suitability

For system suitability, six replicates of the working standard sample were injected and the parameters like plate number (N), peak symmetry and resolution of samples were calculated. These results are shown in table.8.

Table 8 System suitability parameters for the proposed method

Parameters	Aliskiren	Amlodipine	*Hydrochlorothiazide*
Retention time	6.4	5.8	*2.6*
Tailing factor	1.24	1.21	*1.17*
Theoretical plates	4137	4137	*4137*
Resolution	--------	*5.9*	*5.05*

Specificity and selectivity of the proposed method

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Estimation of drugs from tablet dosage forms

Ten tablets of "Amturnide" (Novartis pharma pvt,Ltd, Mumbai, India.) were weighed and ground to a fine powder. From this, an amount equivalent to about 25 mg of lamivudine was transferred into a 100 mL volumetric flask and to it 60 mL of methanol was added and sonicated for 20 min. The volume was made up with methanol. A portion of this solution was filtered through a 0.22 µm membrane filter (discarding the first few mL of the filtrate). 1.0 mL of this filtrate was transferred into a 10 mL volumetric flask containing 5 mL of the diluent. The volume was made up with the diluent and mixed well to get final concentrations of 300 µg/mL, 10µg/mL and 25 µg/mL of Aliskiren, Amlodipine and Hydrochlorothiazide respectively. This solution was chromatographed six times. The mean peak areas of the drugs were calculated and the drug content in the formulation was calculated. A typical chromatogram obtained from the analysis of "Amturnide" tablet is shown in the Fig. 7.

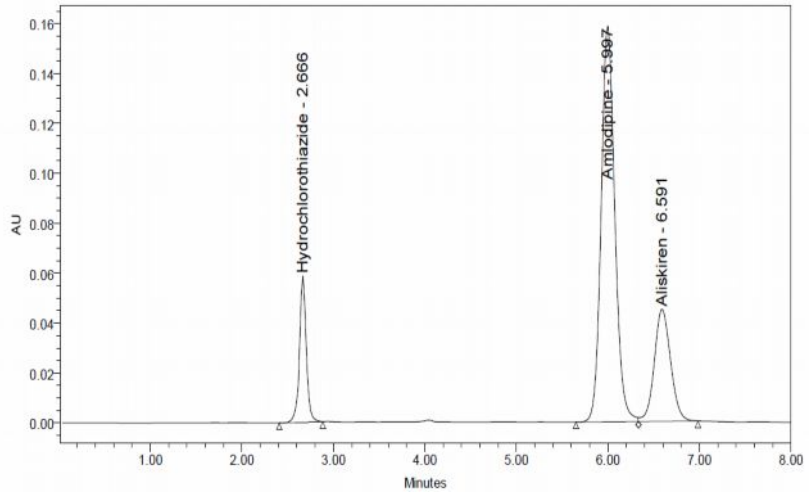

Fig 7 Typical Chromatogram for the separation of Aliskiren, Amlodipine and Hydrochlorothiazide from the formulation sample solution method suitability

Method suitability

The commercial tablet formulation, ""Amturnide"" (Novartis pharma pvt,Ltd, Mumbai, India.) was analyzed by the proposed method and the results are shown in Table 9. The values were found to be in good agreement with the labeled amounts, which confirms the suitability of the method for the analysis of drugs in pharmaceutical dosage forms.

Table 9.Recovery of the drugs from the tablet dosage form "Amturnide"

Drug	Labeled amount (mg)	Amount recovered (mg)	% Recovery
Aliskiren	300	299	99.66
Amlodipine	10	9.9	98.00
Hydrochlorothiazide	25	24.49	99.92

Summary of the Results and Discussion

To optimize the mobile phase, various proportions of the buffer (pH 3.0) and methanol acetonitrile were tested. The use of acetonitrile, methanol and buffer in the ratio of 20:50:30 v/v, pumped at a flow rate of 1.0 mL/min eluted the compounds with short retention times. The corresponding chromatograms showed good baseline stability, peak shape and peak resolution. By applying the proposed method, the retention times of Aliskiren, amlodipine and hydrochlorothiazide were found to be 2.6 min, 5.9 min and 6.5 min respectively. Quantitative linearity was obeyed in the concentration range of 75-450 µg/mL for Aliskiren and 6.25-37.5µg/mL for amlodipine and 2.5-15 µg/mL hydrochlorothiazide respectively. The regression equations of the linearity plots constructed for Aliskiren y = 2598x + 15588 (R^2=0.9996), Amlodipine y = 23204x + 84207(R^2=0.9996) and Hydrochlorothiazide y = 16233x + 10423 (R^2=0.9995) respectively. Where y refers to the area of the peak and x refers to the concentration (µg/mL) of the drug. The correlation coefficients were greater than 0.99. The theoretical plate values obtained for Aliskiren, amlodipine and hydrochlorothiazide were 4129, 3588 and 6705 respectively. Since the number of theoretical plates for the three drugs was above 2000, it indicates that the column was efficient in separating all the three drugs. The limit of detection and limit of quantitation for Aliskiren, amlodipine and hydrochlorothiazide were found to be 0.1521 and 0.2305 µg/mL, 0.2132 and 0.3015 µg/mL and 0.2517 and 0.2615 µg/mL respectively. These values reveal that the method is sensitive. The high percentage of recovery (99.77-99.37, 99.31) indicates that the proposed method is accurate.

No interfering peaks were found in the chromatograms run for the formulation samples. This indicates that the excipients used in tablet formulations did not interfere with the estimation of the drugs by the proposed HPLC method.

Conclusion

A simple isocratic RP – HPLC method with VU detection has been developed for simultaneous determination of ALN, AMB and HCT. The method was validated for accuracy, precision, specificity and linearity. The run time is relatively short (10 mins), which enables rapid quantification of many samples in routine and quality control analysis of tablets. The method also uses a solvent system with the same composition as the mobile phase for dissolving and extracting drugs from the matrices, thus minimizing noise. Thus the proposed method is rapid, selective, requires a simple sample preparation procedure, Moreover, The lower solvent consumption leads to a cost effective and represents a good procedure of ALN, AMB and HCT determination in bulk and Pharmaceutical dosage forms

The present research method has many of advantages over the preceding reported methods. The retention times of the drugs achieved in this method were shorter than the reported methods. The short run time shows the rapid analysis which enables more number of samples to be analyzed per unit time. The range of quantitation obtained in the current method is wider than some of the reported methods. The present RP-HPLC method is rapid, precise and accurate and can be used for routine quality control analysis for simultaneous determination of Aliskiren, amlodipine and hydrochlorothiazide in their tablet dosage forms.

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