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Development and Validation of a Stability Indicating RP-HPLC Method for Simultaneous Estimation of Aliskiren Hemifumarate, Amlodipine Besylate and Hydrochlorothiazide in Bulk and Pharmaceutical Dosage Forms

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Abstract: *A simple, selective and precise RP-HPLC method was developed for the simultaneous estimation of Aliskiren Hemifumarate, amlodipine besylate and hydrochlorothiazide in the Bulk and Pharmaceutical Dosage Forms using losartan as an internal standard. The chromatographic separation of the three drugs was achieved on a reverse phase Inertsil-ODS, C18, 100X 4.6 mm, 5µm column using 0.1 M Ammonium acetate buffer (pH adjusted to 5 using formic acid) and Acetonitrile in the ratio of 65:35 v/v with flow rate of 1.0 ml/min with injection volume 20 µL and the detection was carried out at 232 nm. The retention time of aliskiren hemifumarate (ALSK), amlodipine besylate (AMLO) and hydrochlorothiazide (HCT) were found to be 3.90, 5.22 and 1.91 min respectively. The drug products were subjected to stress conditions of acidic, alkaline, oxidation, UV and Thermal conditions. The degradation products were well resolved from ALSK, AMLO and HCT peaks, thus indicating the stability-indicating nature of the method. The linear regression analysis data for the calibration plots showed good linear relationship in the concentration range of 37.5-225.00 µg/ml for aliskiren hemifumarate, 3.125-18.75 µg/ml for hydrochlorothiazide and 1.25-7.50 µg/ml for amlodipine besylate. The developed method was successfully validated in accordance to ICH guidelines. Hence, this method can be conveniently adopted for the routine analysis in quality control laboratories.*

Key words: *Aliskiren hemifumarate, Amlodipine besylate, Hydrochlorothiazide, RP-HPLC*

I. Introduction

Aliskiren, (2(S), 4(S), 5(S), 7(S)-N- (2-carbamoyl- 2- methylpropyl) -5-amino-4-hydroxy2,7 diisopropyl-8-[4-methoxy-3-(3-methoxypropoxy)phenyl] octanamide hemifumarate) 1-3 (Fig. 1). The first oral direct renin inhibitor approved for clinical use, exhibits a novel and advantageous pharmacokinetic and pharmacodynamic profile for the long-term treatment of hypertension. Aliskiren blocks the renin system at its rate-limiting step by directly inhibiting the catalytic activity of renin, thereby reducing generation of angiotensin I and angiotensin II.

Amlodipine, 2[(2-aminoethoxy) methyl]-4-(2-chloro-phenyl)-1, 4-dihydro-6-methyl-3, 5-pyridine carboxylic acid, 3-ethyl, 5-methylester is a dihydro pyridine (Fig. 2) derivative with calcium antagonist activity. It is used in the management of hypertension, chronic stable angina pectoris and prinzmetal variant angina. Amlodipine inhibits the trans-membrane influx of calcium ions into vascular smooth muscle and cardiac muscle.

Hydrochlorothiazide belongs to Thiazide class of diuretics, acting on the kidneys to reduce sodium (Na) reabsorption in the distal convoluted tubule. This increases the osmolarity in the lumen, causing less water to be reabsorbed by the collecting ducts. This leads to increase urinary output. It is chemically 6-chloro-1, 1-dichloro-3, 4,dihydro -2H-1, 2, 4-benzoliadiazine-7-sulphanomide1, 1-dioxide (Fig. 3).

Literature survey revealed HPLC[1], LC-MS[2], spectroflurimetric and simultaneous UVspectrophotometric methods are reported for the estimation of aliskiren hemifumarate[3-4], amlodipine[5-10], hydrochlorothiazide[11-15] alone or in combination with other anti-hypertensive agents. So the present study aim to develop a simple, selective and precise RP-HPLC method was developed for the simultaneous estimation of Aliskiren hemifumarate, amlodipine besylate and hydrochlorothiazide in bulk drug and in combined dosage form.

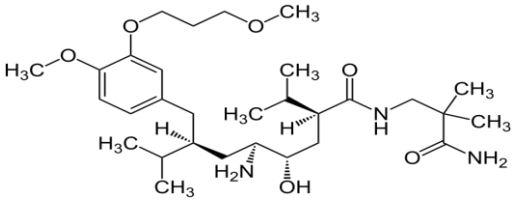

Fig. 1 Structure of Aliskiren

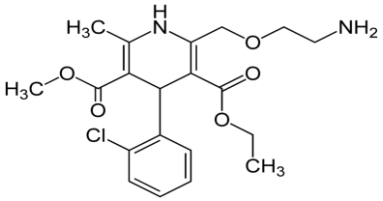

Fig. 2 Structure of Amlodipine

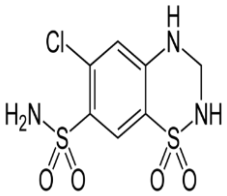

Fig. 3 Structure of Hydrochlorothiazide

II. Materials And Methods

2.1 Chromatographic Conditions

The Waters HPLC with PDA detector and Empower 2 software was employed for the present study. The chromatography determination performed at ambient temperature by using Inertsil-ODS, C18, 100 X 4.6, 5µm) column, with a mobile phase composed of 0.1M ammonium acetate: Acetonitrile in the ratio 65:35 (v/v). pH of the buffer was adjusted to 5 using formic acid. The chromatography run time was maintained up to 10.0 min with flow rate at 1.0 mL / min with injection volume 20µL and the eluent was monitored at 232 nm.

2.2 Reference Standards, Reagents

The standard Aliskiren hemifumarate was obtained from Mylan Pharmaceuticals, Hyderabad. Amlodipine and Hydrochlorothiazide were procured from Life Care Pharmaceutical Industries, Pondicherry. Amturnide tablets were procured from US market. Acetonitrile and water employed for the preparation of mobile phase were of HPLC grade was obtained from Merck limited, Mumbai.

2.3 Preparation of standard solutions

Weigh and transfer 12.5 mg of Hydrochlorothiazide, 150 mg of Aliskiren and 5 mg of Amlodipine working standard into 100 mL volumetric flask, add 50 mL of diluent and sonicate and make up to the volume with diluent. Transfer 10 mL of standard stock solution into 100 mL volumetric flask and dilute it with diluent.

2.4 Preparation of Sample Solutions

20 tablets (Amturnide) were taken and finely powdered. Powder equivalent to 12.5 mg of Hydrochlorothiazide,150 mg of Aliskiren and 5 mg of Amlodipine was taken in to a 100 mL volumetric flask add 50 mL of diluent, sonicate for 10 minutes and make up the volume. Further filter the solution through 0.45 µ membrane filter paper. Dilute 10 ml of filtrate to 100 ml with mobile phase.

2.5 Assay procedure

In case of marketed formulations, twenty tablet were taken and finely powdered. Powder equivalent to 12.5 mg of Hydrochlorothiazide, 150 mg of Aliskiren and 5 mg of Amlodipine were taken in to a 100 mL volumetric flask add 50 mL of diluent, sonicate for 10 minutes and make up the volume.The column was equilibrated for 30min, with the mobile phase flowing through the system with a flow rate of 1.0 ml/min and detector was set at a wavelength of 232nm. The retention time of aliskiren hemifumarate, amlodipine besylate and hydrochlorothiazide were found to be 3.90, 5.22, 1.91min (Fig. 4) respectively in bulk drug and the

retention time of aliskiren hemifumarate, amlodipine besylate and hydrochlorothiazide were found to be 3.90, 5.22, 1.91 min (Fig. 5) respectively in pharmaceutical formulation. The blank chromatogram is shown in Fig. 6. The % purity of aliskiren hemifumarate, amlodipine besylate and hydrochlorothiazide in tablet dosage form were compiled and reported in "Table 1".

Table 1: Determination of ALSK, AMLO and HCT in Tablet dosage form

Drug	Label claim(mg)	Amount found(mg)	Drug content (%)
Aliskiren	150	149.56	99.71
Amlodipine	5	5.05	100.97
Hydrochlorothiazide	12.5	12.48	99.86

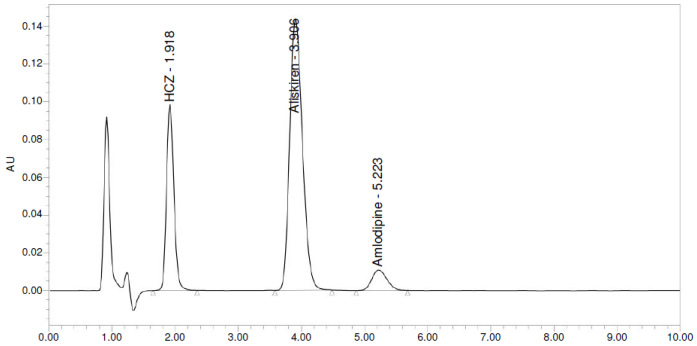

Fig. 4 Standard chromatogram of Aliskiren hemifumarate, Hydrochlorothiazide and Amlodipine

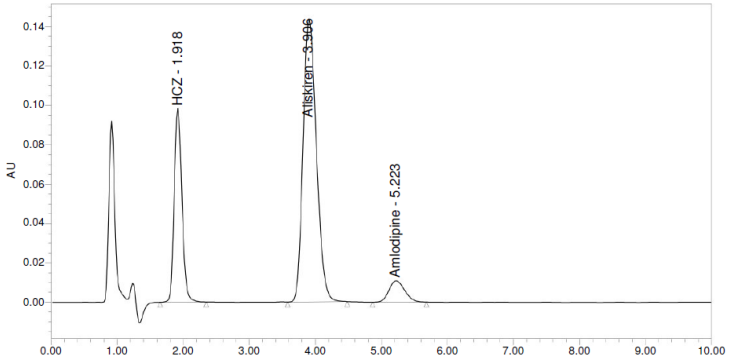

Fig. 5 Sample chromatogram of Aliskiren hemifumarate, Hydrochlorothiazide and Amlodipine

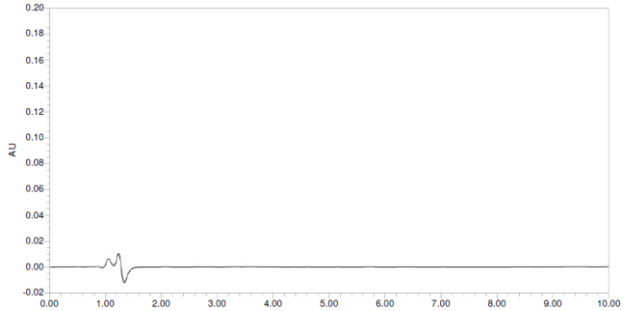

Fig. 6 Chromatogram of Blank

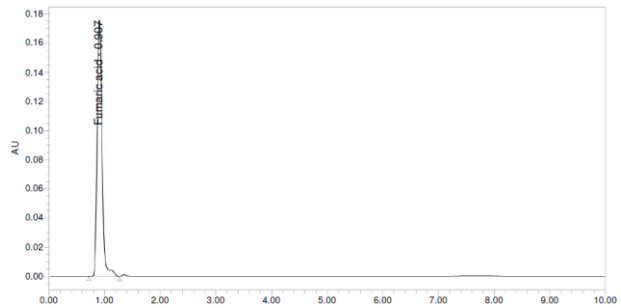

Fig. 7 Chromatogram of Fumaric acid

III. Method Validation

After the method conditions were established as described above, method was validated as per ICH guidelines. The accuracy, precision, Linearity, limit of detection (LOD) and quantification (LOQ) were determined. These values are summarized in Table 10.

3.1 Accuracy

Accuracy was determined in terms of percentage recovery. Sample solution spiked with the analytes at three different concentration levels 37.5-225.00µg/ml of Aliskiren hemifumarate, 3.125-18.75 µg/ml of Hydrochlorothiazide and 1.25-7.50µg/ml of Amlodipine. Another set of standard mixtures at the same concentration levels were also prepared with the diluents. Sample and standard solutions were injected into the HPLC system. Percentage recoveries of Aliskiren hemifumarate, Hydrochlorothiazide and Amlodipine were calculated. The values are summarized in Table 2.

Table 2: Recovery Studies

Drug	Concentration	Peak area (avg)	Amount of drug added	Amount of drug found	% Recovery
Aliskiren	80%	1611146	120.10	120.16	100.05
	100%	2025916	150.15	151.09	100.60
	120%	2425139	180.90	180.86	99.98
Amlodipine	80%	140674	6.05	6.06	99.67
	100%	178060	5.08	5.13	100.93
	120%	213714	4.00	3.99	100.11
Hydrochlorothiazide	80%	646293	10.01	10.09	100.84
	100%	805837	12.50	12.59	100.69
	120%	961011	15.02	15.01	99.93

3.2 Precision

Method precision was determined both in terms of repeatability (injection and analysis) and intermediate precision (intra-day and inter-days reproducibility). In order to determine injection repeatability, samples spiked with Aliskiren hemifumarate, hydrochlorothiazide and amlodipine were injected 6 times into HPLC system and repeatability of the retention time and peak area were determined and expressed as mean and %RSD calculated from the data obtained. The values are summarized in Table 3, 4, 5 and 6.

Table 3: System Precision

Name	Hydrochlorothiazide		Aliskiren		Amlodipine	
	RT	Area	RT	Area	RT	Area
System Precision-1	1.916	799421	3.896	2014646	5.207	177004
System Precision-2	1.912	794230	3.905	2002840	5.216	176608
System Precision-3	1.919	796920	3.907	2006750	5.222	178796
System Precision-4	1.915	797648	3.904	2017752	5.223	178685
System Precision-5	1.918	797648	3.906	2017752	5.223	178685
System Precision-6	1.914	794230	3.905	2002840	5.216	176608
Avg	1.916	796683	3.904	2010430	5.218	177731
Std Dev	0.003	2071.22	0.004	7124.00	0.006	1095.92
RSD	0.135	0.260	0.102	0.354	0.120	0.617

Table 4: Method Precision

Name	Hydrochlorothiazide		Aliskiren		Amlodipine	
	RT	Area	RT	Area	RT	Area
Method Precision-1	1.912	796525	3.905	2006841	5.222	178796
Method Precision-2	1.916	797562	3.905	2017774	5.224	178692
Method Precision-3	1.912	794241	3.906	2010845	5.212	176625
Method Precision-4	1.915	797572	3.905	2016785	5.226	178794
Method Precision-5	1.916	797742	3.904	2017854	5.225	178881
Method Precision-6	1.918	797644	3.904	2017651	5.223	178781
Avg	1.915	796881	3.905	2014625	5.222	178428
Std Dev	0.002	1368.29	0.001	4669.99	0.005	885.40
RSD	0.125	0.172	0.019	0.232	0.098	0.496

Table 5: Interday Precision

Name	Hydrochlorothiazide		Aliskiren		Amlodipine	
	RT	Area	RT	Area	RT	Area
Injection -1	1.916	799421	3.896	2014646	5.207	177004
Injection -2	1.912	794230	3.905	2002840	5.216	176608
Injection -3	1.919	796920	3.907	2006750	5.222	178796
Injection -4	1.915	797648	3.904	2017752	5.223	178685
Injection -5	1.918	797648	3.906	2017752	5.223	178685
Injection -6	1.914	794230	3.905	2002840	5.216	176608
Avg	1.916	796683	3.904	2010430	5.218	177731
Std Dev	0.003	2071.22	0.004	7124.00	0.006	1095.92
RSD	0.135	0.260	0.102	0.354	0.120	0.617

Table 6: Intraday Precision

Name	Hydrochlorothiazide		Aliskiren		Amlodipine	
	RT	Area	RT	Area	RT	Area
Injection -1	1.912	794465	3.901	2024665	5.211	178012
Injection -2	1.912	796241	3.908	2012651	5.221	176712
Injection -3	1.915	795845	3.904	2026541	5.220	178825
Injection -4	1.918	796651	3.905	2018742	5.208	178672
Injection -5	1.913	798045	3.908	2018754	5.212	178665
Injection -6	1.915	798625	3.902	2012856	5.215	177654
Avg	1.914	796645	3.905	2019035	5.215	178090
Std Dev	0.002	1512.35	0.003	5781.65	0.005	812.97
RSD	0.121	0.190	0.075	0.286	0.099	0.456

3.3 Linearity

The linearity of the method was established by spiking a series of standard mixtures of Aliskiren hemifumarate (37.5-225.00µg/ml), Hydrochlorothiazide (3.125-18.75µg/ ml) and Amlodipine (1.25-7.50µg/ml) and the above solutions are injected onto the HPLC system. The standard Calibration curve for Aliskiren hemifumarate (Fig. 8), Amlodipine (Fig. 9) and Hydrochlorothiazide (Fig. 10) was constructed by plotting their response ratios (ratios of the peak area of the analytes) against their respective concentrations. Linear regression was applied and slopes (a), intercept (b), correlation coefficient (r) were determined. The values are summarized in Table 7.

Table 7: Linearity

Sl. no.	Aliskiren (µg/ml)	Area	Amlodipine (µg/ml)	Area	HCT (µg/ml)	Area
1.	37.50	527267	1.25	44451	3.125	212852
2.	75.00	1014161	2.50	89006	6.250	405379
3.	112.50	1512403	3.75	132692	9.375	602830
4.	150.00	2005341	5.00	175598	12.500	795096
5.	187.50	2507191	6.25	221176	15.625	986734
6.	225.00	3025116	7.50	263987	18.750	1184882

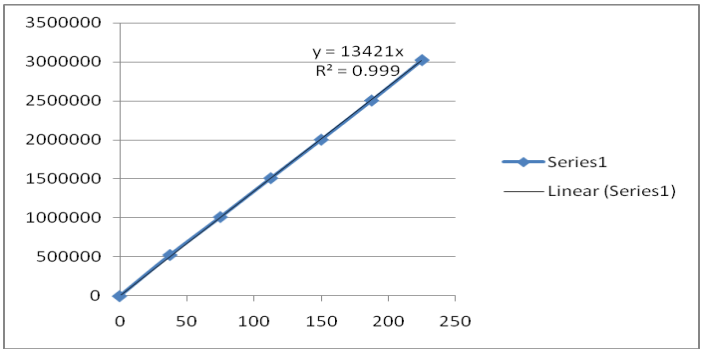

Figure 8: Standard calibration graph of Aliskiren

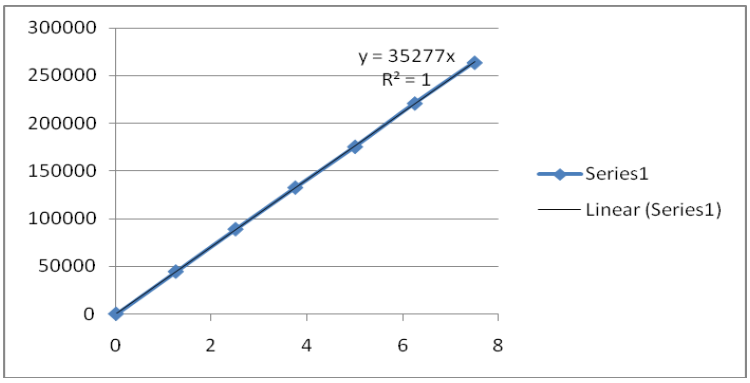

Figure 9: Standard calibration graph of Amlodipine

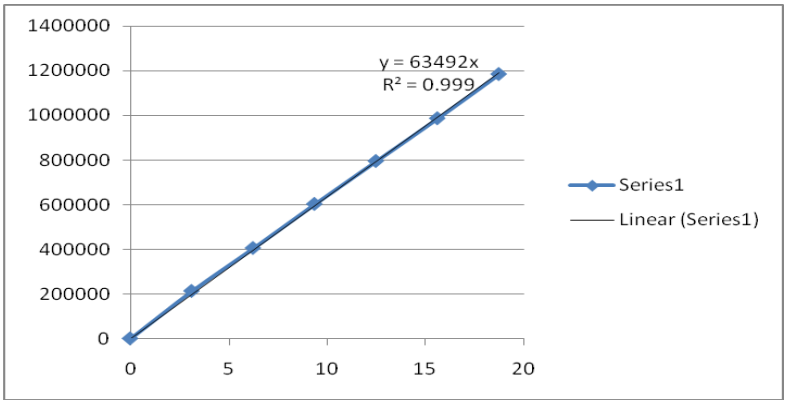

Figure 10: Standard calibration graph of Hydrochlorothiazide

3.4 Limit of detection & Limit of quantification

Detection and quantification limits were determined through dilution method using S/N approach by injecting a 20µl sample. LOD was considered as the minimum concentration with a signal to noise ratio of atleast three (S/N˜3), while LOQ was taken as a minimum concentration with a signal to noise ratio of atleast ten (S/N˜10). The LOD for Aliskiren, Hydrochlorothiazide, Amlodipine standard solutions were found to be 2.886 ng/ml , 2.98 ng/ml and 2.99ng/ml respectively. The LOQ for Aliskiren, Hydrochlorothiazide, Amlodipine standard solutions were found to be 9.79ng/ml, 10.001ng/ml and 9.98ng/ml respectively (Table 10).

3.5 Robustness

As defined by ICH, The robustness of an analytical procedure describes to its capability to remain unaffected by small and deliberate variations in method parameters. Robustness was performed by small variation in the chromatographic conditions and found to be unaffected by small variations like flow rate (±10%), column oven temperature (±5°c) and wave length (±5 units). The values are summarized in Table 8.

Table 8: Robustness

Drug	Flow rate	Column oven temperature	Wavelength
Aliskiren	0.9 ml 1.1 ml	25°c 35°c	227nm 237nm
Amlodipine	0.9 ml 1.1 ml	25°c 35°c	227nm 237nm
Hydrochlorothiazide	0.9 ml 1.1 ml	25°c 35°c	227nm 237nm

3.6 Ruggedness

The method is rugged by different analyst, different time intervals and the method did not significantly affect the recoveries, peak area and retention time of all the above drugs indicating that the proposed method is rugged. The values are summarized in Table 9.

Table 9: Ruggedness

Name	Hydrochlorothiazide		Aliskiren		Amlodipine	
	RT	Area	RT	Area	RT	Area
Injection -1	1.912	794465	3.901	2024665	5.211	178012
Injection -2	1.912	796241	3.908	2012651	5.221	176712
Injection -3	1.915	795845	3.904	2026541	5.220	178825
Injection -4	1.918	796651	3.905	2018742	5.208	178672
Injection -5	1.913	798045	3.908	2018754	5.212	178665
Injection -6	1.915	798625	3.902	2012856	5.215	177654
Avg	1.914	796645	3.905	2019035	5.215	178090
Std Dev	0.002	1512.35	0.003	5781.65	0.005	812.97
RSD	0.121	0.190	0.075	0.286	0.099	0.456

Table 10: Validation Parameters of the Method

Method Parameters	Aliskiren	Amlodpine	Hydrochlorothiazide
Linearity range (µg/ml)	37.5-225.00	1.25-7.50	3.125-18.75
Correlation coefficient	0.999	1	0.999
LOD (ng/ml)	2.88	2.99	2.98
LOQ (ng/ml)	9.79	9.98	10.001
Retention time	3.90	5.22	1.91
Theoretical plates	3574	3454	3274
Tailing factor	1.16	1.14	1.12
Resolution	1.68	3.21	5.20
Precision (%RSD)	0.286	0.456	0.190
Intra-day (n=3)	0.075	0.099	0.121
Inter-day (n=3)	0.102	0.120	0.135
% Recovery (n=6)	100.21	100.23	100.42

IV. Forced Degradation Studies (Stress Testing)

Forced degradation studies were carried out for all the three drugs. The bulk drugs were subjected to alkaline studies by adding 1.0 ml of 0.1M NaOH for 4hrs, 8hrs and 12hrs neutralized with 1.0 ml of 0.1M HCl acid. Similarly, the acidic studies were performed by adding 1.0 ml of 0.1 M HCl for 4hrs, 8hrs and 12hrs and neutralized with 1ml of 0.1M NaOH. Oxidation studies were performed on bulk drug by adding 1.0 ml of 3% H_2O_2, thermal studies were performed by keeping the drug at 100˚C and UV studies were performed with UV-Lamp for 4hrs, 8hrs and 12hrs respectively (Figure 11 – 15). All samples were taken in different 10 ml volumetric flask and dissolved in mobile phase. Final assay drug concentration was made up with mobile phase and injected in the chromatographic system. For all the stability study, the formation of degradable product was confirmed by comparing to chromatogram of the solution kept under normal conditions. All stressed samples were analyzed by developed HPLC method. The degradation data for Aliskiren hemifumarate, Amlodipine besylate and Hydrochlorothiazide was shown in Table 11, 12 & 13 respectively.

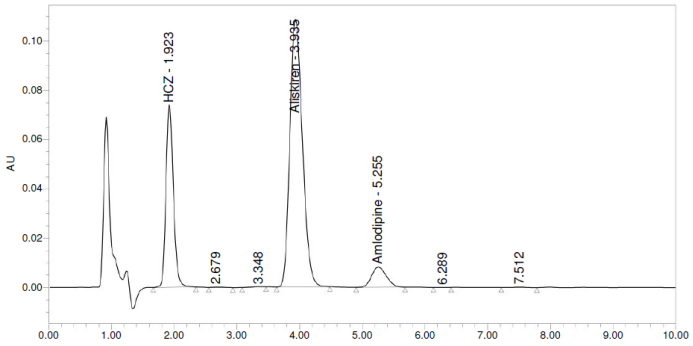

Figure 11: Typical chromatogram of Acid hydrolysis

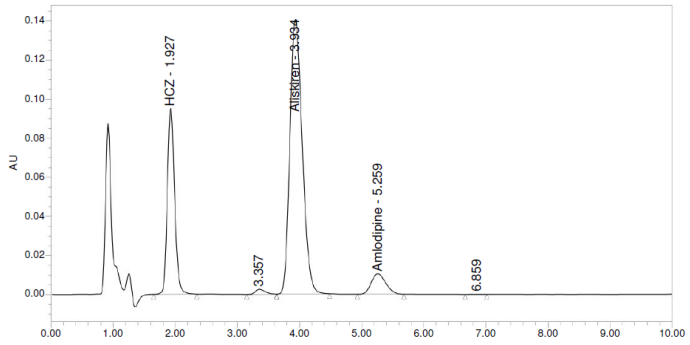

Figure 12: Typical chromatogram of Alkaline hydrolysis

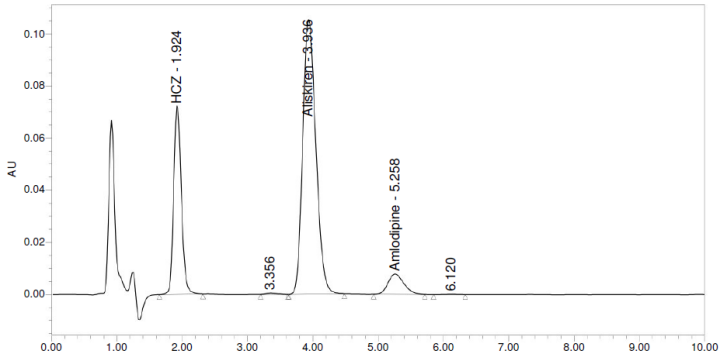

Figure 13: Typical chromatogram of Oxidation

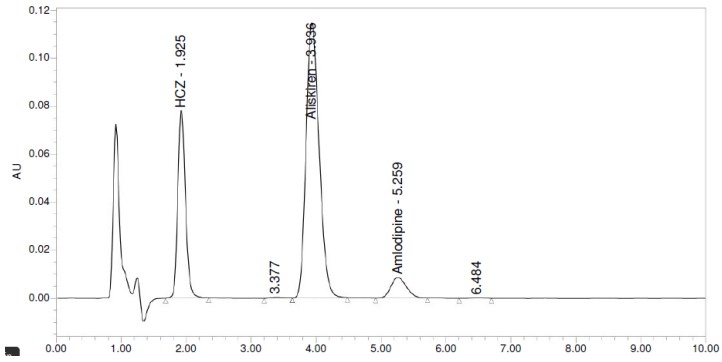

Figure 14: Typical chromatogram of UV

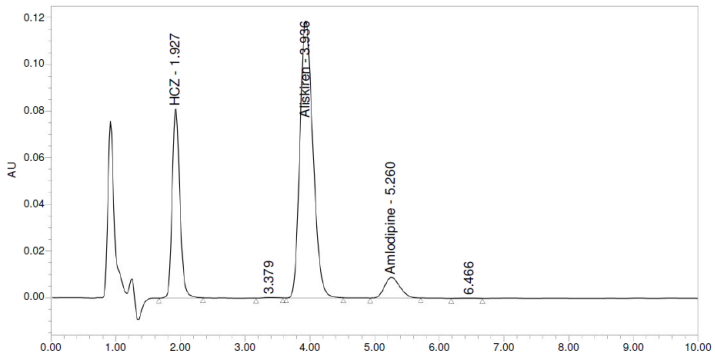

Figure 15: Typical Chromatogram of Thermal Degradation

Table 11: Degradation data for Aliskiren

Stress condition	Degradation time	Degradation (%)
Acidic (0.1Hcl)	4hrs	97.12
	8hrs	93.76
	12hrs	90.17
Alkaline (0.1 NaOH)	4hrs	98.09
	8hrs	97.83
	12hrs	95.06
Oxidation (H_2O_2)	4hrs	95.36
	8hrs	89.14
	12hrs	83.09
UV	4hrs	96.89
	8hrs	92.37
	12hrs	88.99
Thermal	4hrs	96.17
	8hrs	93.67
	12hrs	87.54

Table 12: Degradation data for Amlodipine

Stress condition	Degradation time	Degradation (%)
Acidic (0.1Hcl)	4hrs	97.38
	8hrs	95.18
	12hrs	92.77
Alkaline (0.1 NaOH)	4hrs	98.9
	8hrs	97.71
	12hrs	95.17
Oxidation (H_2O_2)	4hrs	98.09
	8hrs	94.53
	12hrs	90.26
Thermal	4hrs	97.22
	8hrs	95.62
	12hrs	93.41
UV	4hrs	96.48
	8hrs	91.32
	12hrs	89.61

Table 13: Degradation data for Hydrochlorothiazide

Stress condition	Degradation time	Degradation (%)
Acidic (0.1Hcl)	4hrs	96.73
	8hrs	92.59
	12hrs	88.83
Alkaline (0.1 NaOH)	4hrs	99.12
	8hrs	98.01
	12hrs	96.00
Oxidation (H_2O_2)	4hrs	95.49
	8hrs	91.23
	12hrs	86.48
Thermal	4hrs	96.84
	8hrs	92.17
	12hrs	87.56
UV	4hrs	97.36
	8hrs	94.91
	12hrs	91.96

V. Conclusion

A novel, simple, rapid and cost effective RP-HPLC method was successfully developed for simultaneous determination of Aliskiren Hemifumarate, amlodipine besylate and hydrochlorothiazide. The proposed method was optimized and validated for the various experimental parameters. Influence of pH of the mobile phase, column oven temperature and various particulate columns on the analysis of Aliskiren Hemifumarate, amlodipine besylate and hydrochlorothiazide was evaluated. All the analytes were well resolved and separated in less than 10 min. The developed method is a stability indicating method and can be conveniently used by quality control outfits to determine the contents of Aliskiren Hemifumarate, amlodipine besylate and hydrochlorothiazide simultaneously in routine and stability samples. This method could be used for the analysis of the drugs in pharmaceutical preparations and routine laboratory analysis with slight modification in the extraction procedure. Overall, the proposed method provides high throughput for simultaneous determination of Aliskiren Hemifumarate, amlodipine besylate and hydrochlorothiazide with excellent accuracy, precision, selectivity and reproducibility.

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