RP-HPLC Method Development and Validation for Determination of Amiloride and hydrochlorothiazide Bulk Drug Substance and Pharmaceutical Dosage Forms

[1]Dr.A.M.Tamboli, [2]Sourabh Rajendra Bhosale, [3]Vinay Shivaji Kamble, [4]Swapnil Bajirao Hole
Sahyadri College of Pharmacy Methwade

Abstract: A simple, precise, accurate RP-HPLC method with PDA detector has been developed and subsequently validated for the simultaneous estimation of Amiloride and hydrochlorothiazide.in pure and pharmaceutical dosage form. The estimation was carried out on Phenomenex C_8 column (150 x 4.6 mm, 5 um) column, at 1 ml/min flow rate, detection wavelength is 264 nm, mobile phase containing 0.1% Formic acid: Acetonitrile in ratio of 40:60. The retention times of Amiloride and hydrochlorothiazide were 1.47 min & 3.47 min respectively. The concentration range was found to be linear 0.8-1.2 µg/ml for Amiloride and 08-12 µg/ml for hydrochlorothiazide. The correlation coefficient (r^2) was found to be 0.999 for both the drugs. LOD and LOQ value for Amiloride was found to be 0.03 µg/mL and 0.10 µg/mL, LOD and LOQ value for Hydrochlorothiazide was found to be 0.24 µg/mL and 0.72 µg/mL respectively. The % RSD values were less than 2 for both the drugs. The assay of Amiloride and hydrochlorothiazide was found to be 99.66 % and 99.45 % respectively. The method was validated for linearity, accuracy, precision, robustness, LOD, LOQ as per ICH guidelines. The developed method was successfully used for the quantitative analysis of commercial available dosage form.

INTRODUCTION

Amlodipine is synthetically 3-O-ethyl 5-O-methyl 2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)- 6-methyl-1,4-dihydropyridine-3,5-dicarboxylate and is a well known antihypertensive medication having a place with the gathering of medications called dihydropyridine calcium channel blockers[1].

Dihydropyridine calcium channel blockers are associated with a decreased incidence of myocardial depression and cardiovascular conduction deviations from the norm compared to other calcium channel blockers because of their selectivity for the fringe veins. It is typically used to treat angina and high blood pressure. Amlodipine can enhance the production of nitric oxide (NO), a powerful vasodilator that lowers blood pressure, and has anti-cancer properties [2,3].

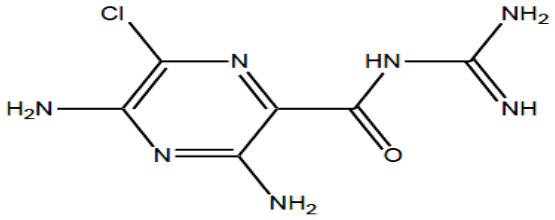

Figure no. 1: Chemical structure of Amiloride

Hydrochlorothiazide is a benzothiadiazine that is 3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide subbed by a chloro bunch at position 6 and a sulfonamide at 7. It is diuretic utilized for the treatment of hypertension and congestive heart failure[4]. It functions as an ecological poison, a diuretic, an antihypertensive specialist, and a xenobiotic. It is an organochlorine, sulfonamide, and benzothiadiazine molecule. The thiazide diuretic that is most frequently prescribed is hydrochlorothiazide. It has been demonstrated to alleviate hypertension and edema. The usage of hydrochlorothiazides is common but is decreasing as angiotensin switches to catalyst inhibitors. Numerous mix items are accessible containing hydrochlorothiazide and angiotensin changing over protein inhibitors or angiotensin II receptor blockers[5].

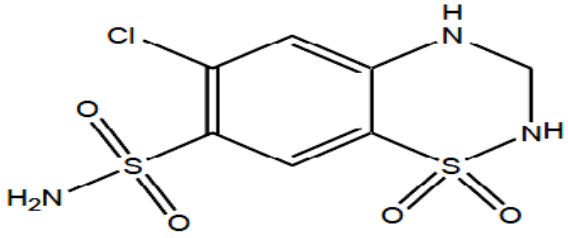

Figure no. 2: Chemical structure of Hydrochlorothiazide

There are few analytical methods for the determination of hydrochlorothiazide alone or in its combination with other drugs in pharmaceutical preparations including spectrophotometry[6,7], TLC[8], and HPLC [9-17]. Hence, the developed method aims in separating the selected drugs simultaneously. The developed method was validated as per the ICH guideleines[18-19].

MATERIAL AND METHODS

1. Chromatographic Conditions:
a. Oven Temp: 30°C
b. Flow rate: 1 ml/min.
c. Mobile Phase: 0.1% Formic acid: Acetonitrile(40: 60, % v/v)
d. Runtime: 7 minutes
e. Injection Volume: 10µl
f. Wavelength: 264nm
g. Diluent: 0.1% Formic acid: Acetonitrile (50: 50, % v/v)
h. Column: Phenomenex Kinetex XB-C8 (150 x 4.6 mm, 5µ)

2. Preparation of Formic acid: In 1000 ml HPLC water, 1.0 ml of Formic acid was added and mixed well and filtered through 0.45-micron membrane filter and sonicated to degas for 10 minutes.
3. Standard Preparation:
a. Amiloride Standard Stock solution-I (SSS-I):
Prepare a Standard Stock Solution (SSS-I) of by adding10mg of Amiloride in 100 ml volumetric flask & add 50 ml diluent, sonicate for 5 minutes and make the volume to 100 ml with diluent. Further 1.0 ml of above solution was transferred in 10 ml volumetric and made up to the mark with diluent and mixed well.(Conc. of Amiloride in SSS-I = 10 µg/ml).

b. Hydrochlorthiazide Standard Stock solution-II (SSS-II)
Prepare a Standard Stock Solution (SSS-II) of by adding 10mg of Hydrochlorthiazide in 10 ml volumetric flask & add 5 ml diluent, sonicate for 5 minutes and make the volume to 10 ml with diluent. Further 1.0 ml of above solution was transferred in 10 ml volumetric and made up to the mark with diluent and mixed well. (Conc. of Hydrochlorthiazide SSS-II = 100 µg/ml).

c. Then add 1.0 ml of SSS-I and 1.0 ml of SSS-II in 10 ml volumetric flask and add 5 ml diluent and vortex and make up the volume with diluent. (Conc. of Amiloride=1 µg/ml and Conc. of Hydrochlorthiazide = 10 µg/ml)

4. Preparation of Drug Product sample solution:
The drug product sample solution was prepared by taking 10 tablets and they were crushed using mortar & pestle and powder equivalent to 1 mg of Amiloride and 10 mg of Hydrochlorthiazide accurately in 100 ml volumetric flask and 50-70diluent was added to it and sonicated for 5 minutes and made up to the mark with diluent.

5. Selection of Wavelength:
The sample was scanned from 190-400 nm with DAD detector. The Wavelength selected for analysis chosen was 264nmon the basis of isobestic point.

6. Method Validation:
a. Specificity & Assay:
i) Individual sample of Blank, Amiloride working standard (1 µg/ml), Hydrochlorthiazide working standard (10 µg/ml), Mixture working standard and Drug product of was prepared and peaks were for identified from Retention Time.

1. % Assay was calculated as follows:

$$\% \text{ Assay} = \frac{\text{Sample area}}{\text{Standard area}} \times 100$$

b. Repeatability& System Suitability:
i. A single working standard was prepared as described in section 2 and 6 injections were made from same solution and checked for system suitability.

ii. System suitability parameters are as below:
1. Retention Time,
2. Theoretical plates,
3. Asymmetry (Tailing factor),
4. Resolution.

c. Linearity & Range:
i) 5 samples of varying concentrations ranging from 80-120% were prepared.
ii) The concentrations are given below
Table no.: 1. Concentration for linearity Study of For HPLC

% Level	Amiloride Conc. (µg/ml)	Hydrochlorthiazide Conc. (µg/ml)
80	0.8	8
90	0.9	9

100	1.0	10
110	1.1	11
120	1.2	12

iii. The sample preparations are given as below;
X ml of Amiloride and Y ml of Hydrochlorthiazide standard solution was added to 10 ml diluent to make up the concentrations given above:

X ml of SSS-I	X ml of SSS-II	Diluted to
0.8	0.8	10 ml
0.9	0.9	10 ml
1.0	1.0	10 ml
1.1	1.1	10 ml
1.2	1.2	10 ml

b. Accuracy:

1. Samples were prepared of 80%, 100% and 120% concentration by spiking the same amount of concentration given in table for Linearity.
2. Samples were injected in triplicate to calculate % RSD.
3. % Recovery was also calculated.

c. LOD/ LOQ:

1. Was calculated by using ANOVA technique.
2. Formula:

$$\text{LOD} = \frac{3.3 \times \text{Std. Error of Intercept}}{\text{Coefficients of X Variable 1}}$$

$$\text{LOQ} = \frac{10 \times \text{Std. Error of Intercept}}{\text{Coefficients of X Variable 1}}$$

d. Robustness:

i. The Robustness was performed by changing the column temperature and Wavelength by ± 2˚C and ± 2 nm.

ii. Each Sample was injected and % RSD of peak area was calculated at each condition.

Table no. 2 Column Oven Temperature Robustness Study.

Condition	Increased	Normal	Decreased
Column Oven Temperature	32˚C	30˚C	28˚C
Wavelength	266 nm	264 nm	262 nm

e. Intra & Inter-day Precision:

i) Single mixture working standard and drug product was prepared and injected twice in a day at different time intervals to evaluate intra-day precision.

ii) Same mixture working standard was analysed on second day to evaluate the inter-day precision.

iii) % RSD of peak was calculated at each interval and stability of solutions were estimated.

RESULT AND DISCUSSION

i) Selection of analytical wavelength:

The sample was scanned from 200-400 nm with PDA detector. The Wavelength selected for analysis chosen was 264 nm on basis of appropriate intensity of Amiloride.

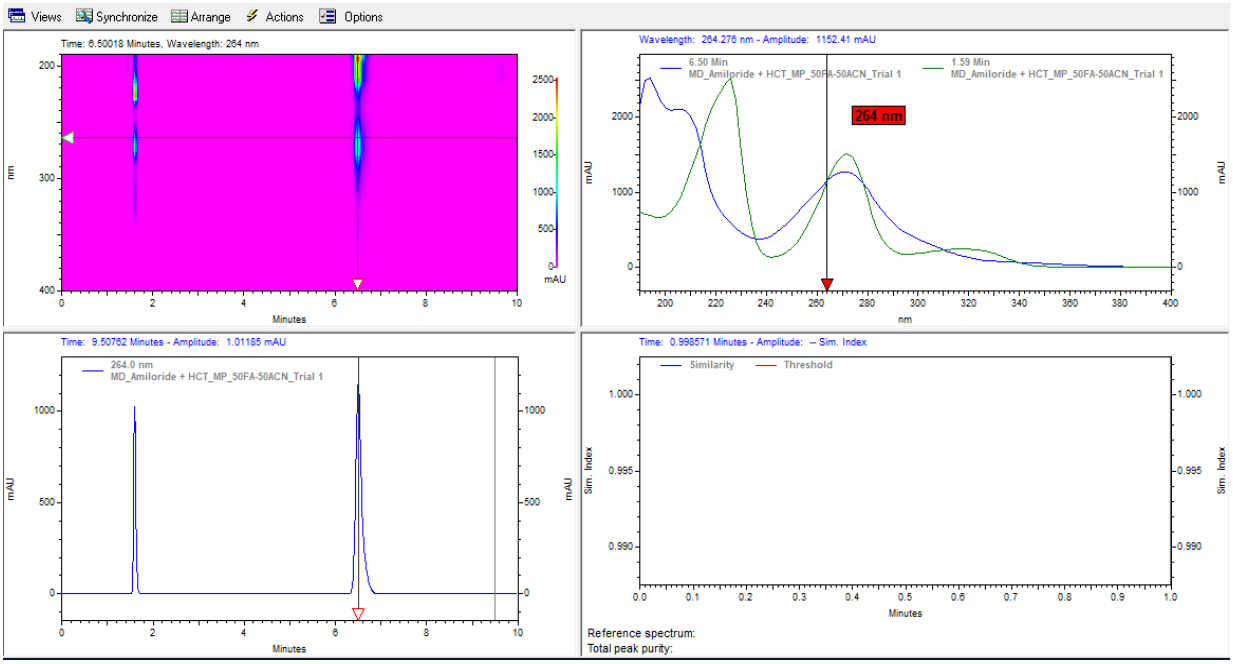

Figure no.3 : Spectrum of Amiloride and Hydrochlorothiazide between 200-400 nm in mobile phase

Amiloride RT 1.47 min and Hydrochlorothiazide RT 3.47 min show the maximum absorbance at 264 nm. Hence, HPLC analysis was carried out at 264 nm. (Figure.1)

Table no. 1 Details of Various trial of mobile phase for mixture containing Amiloride and Hydrochlorothiazide.

Trial No.	Mobile Phase	Ratio	Wavelength	Amiloride		Hydrochlorothiazide		
				RT	TP	RT	TP	Resolution
1	0.1% Formic acid : Acetonitrile	50-50	210 nm	1.59	5012	6.50	12320	30.26
2	0.1% Formic acid : Acetonitrile	45-55	264 nm	1.51	4797	4.31	12178	22.94
3	0.1% Formic acid : Acetonitrile	40-60	264 nm	1.47	4615	3.47	11542	18.51

Final Method: Phenomenex C_8 column (150x4.6 mm, 5 um) column, at 1 ml/min flow rate, detection wavelength is 264 nm, mobile phase containing 0.1% Formic acid: Acetonitrile in ratio of 40:60.

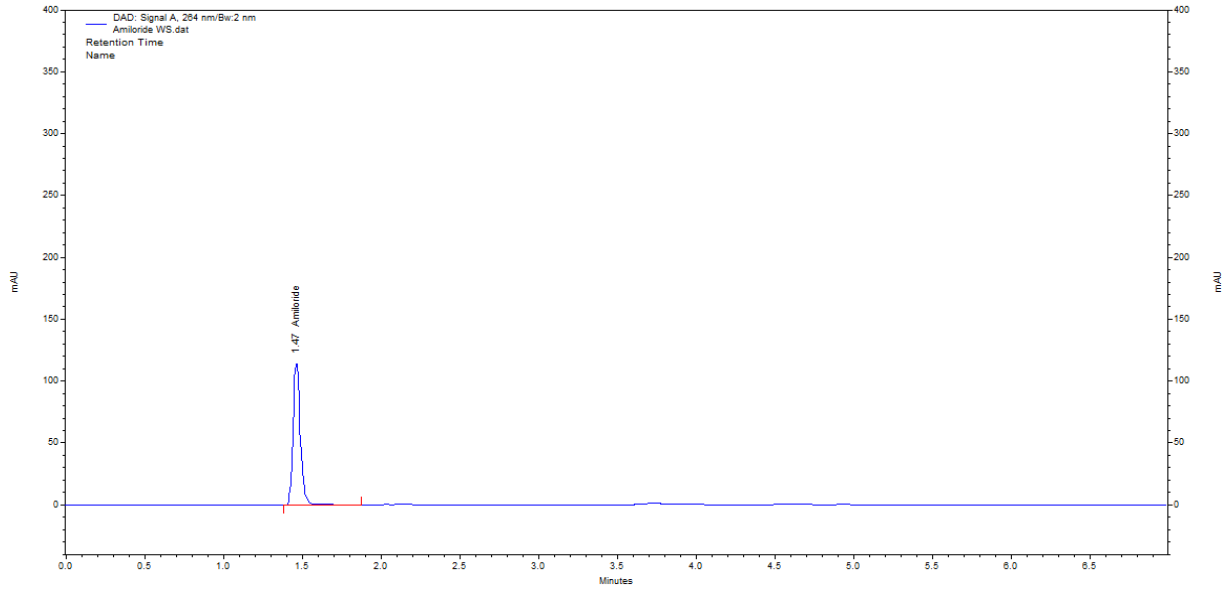

Figure no.4: Chromatogram of Standard Amiloride.

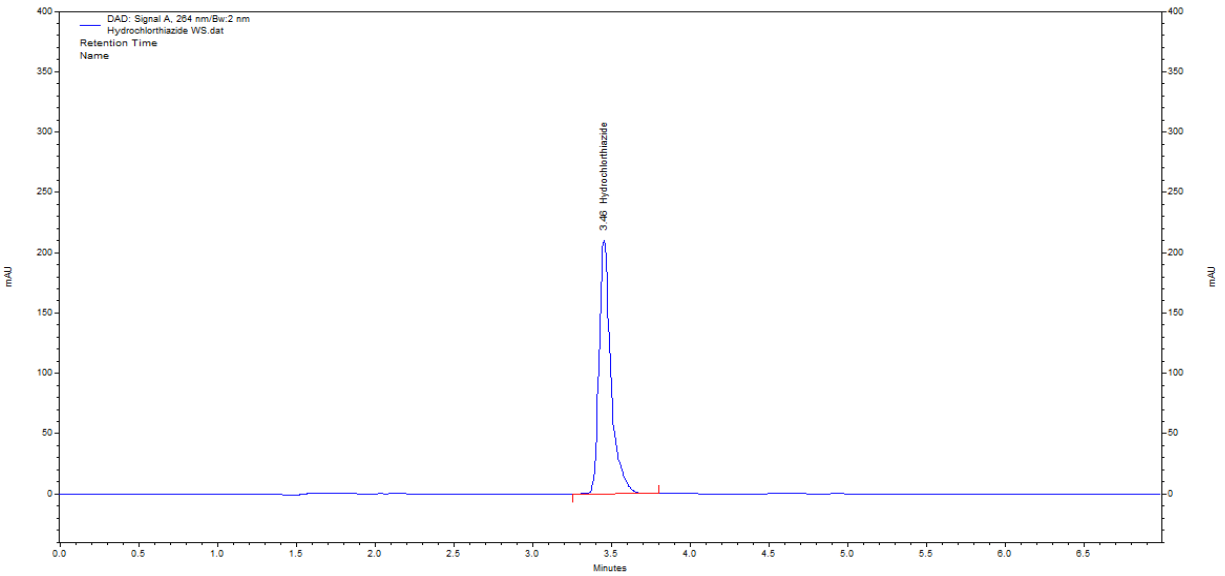

Figure no.5: Chromatogram of Standard Hydrochlorothiazide.

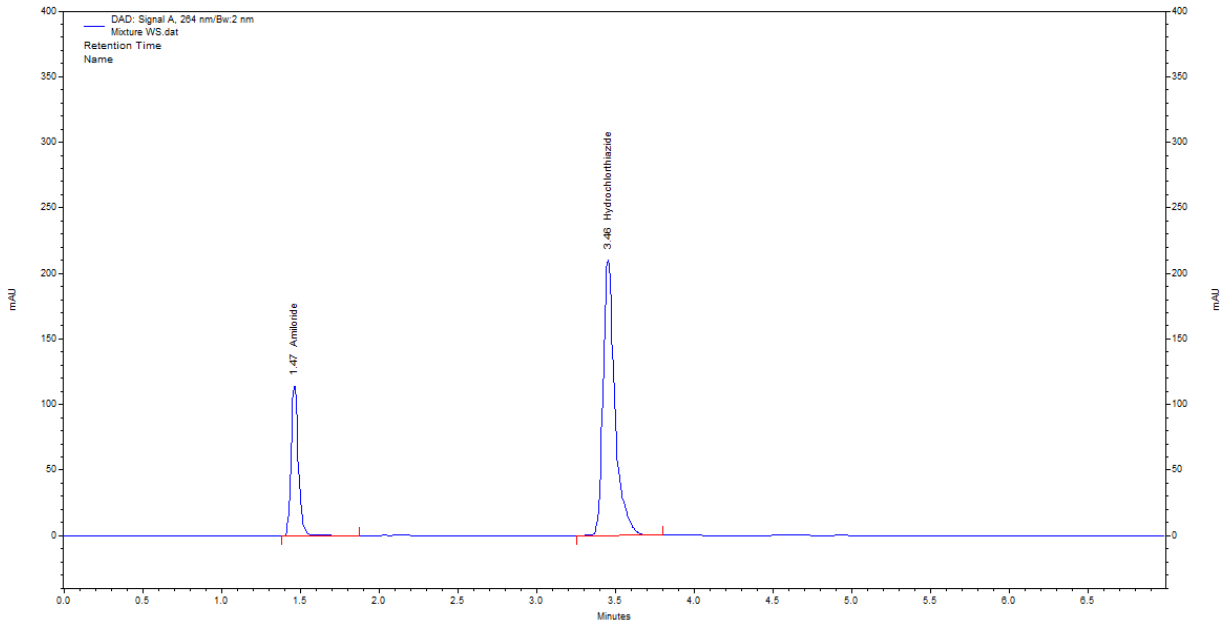

Figure no.6: Chromatogram of Standard Mixture of Amiloride and Hydrochlorothiazide in optimized chromatographic conditions.

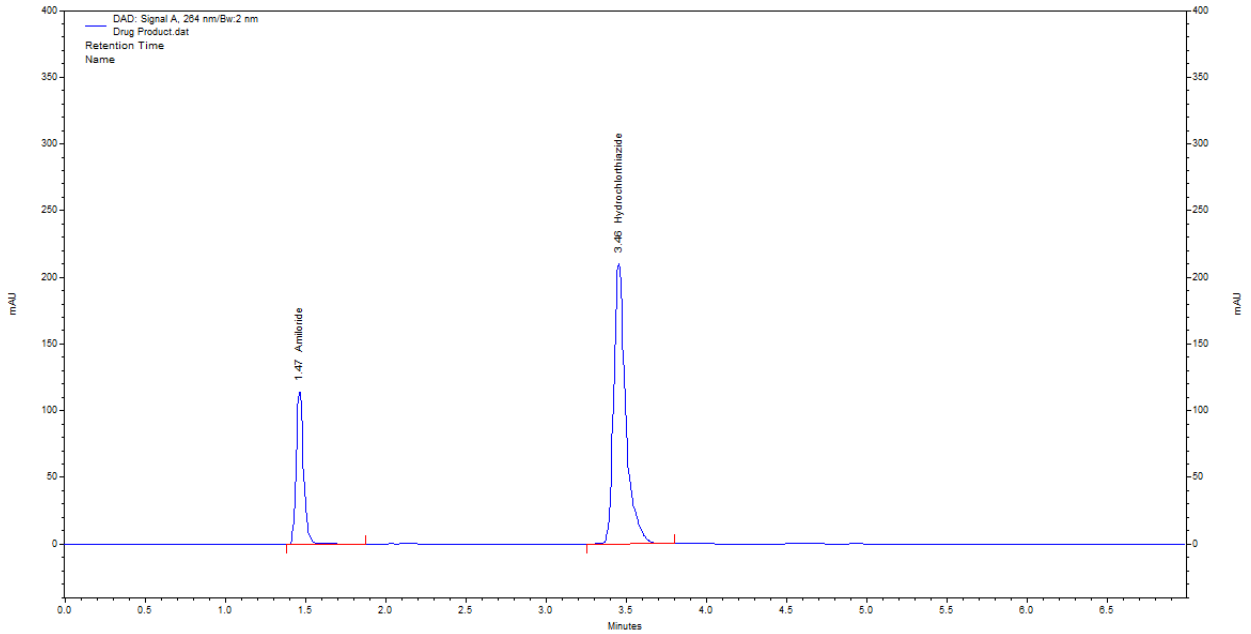

Figure no.7: Chromatogram of Sample of Amiloride and Hydrochlorothiazide in optimized chromatographic conditions.

Table no. 2: Details of chromatogram of standard mixture containing Amiloride and Hydrochlorothiazide.

Sr. No	Name of drug	RT (min)	Plates	Tailing factor
1.	Amiloride	1.47 ± 0.5 min	5012	1.30
2.	Hydrochlorothiazide	3.47 ± 0.5 min	12320	1.53

iii) Analysis of tablet formulation:-

Table no.3 Analysis of marketed formulation.

Sample ID	Amiloride			Hydrochlorothiazide		
	RT	Area	% Assay	RT	Area	% Assay
ALR WS	1.47	765227	-	-	-	-
HCT WS	-	-	-	3.46	2325878	-
MIX WS	1.47	763696	-	3.46	2307262	-
Drug Product	1.47	761105	99.66	3.46	2294575	99.45

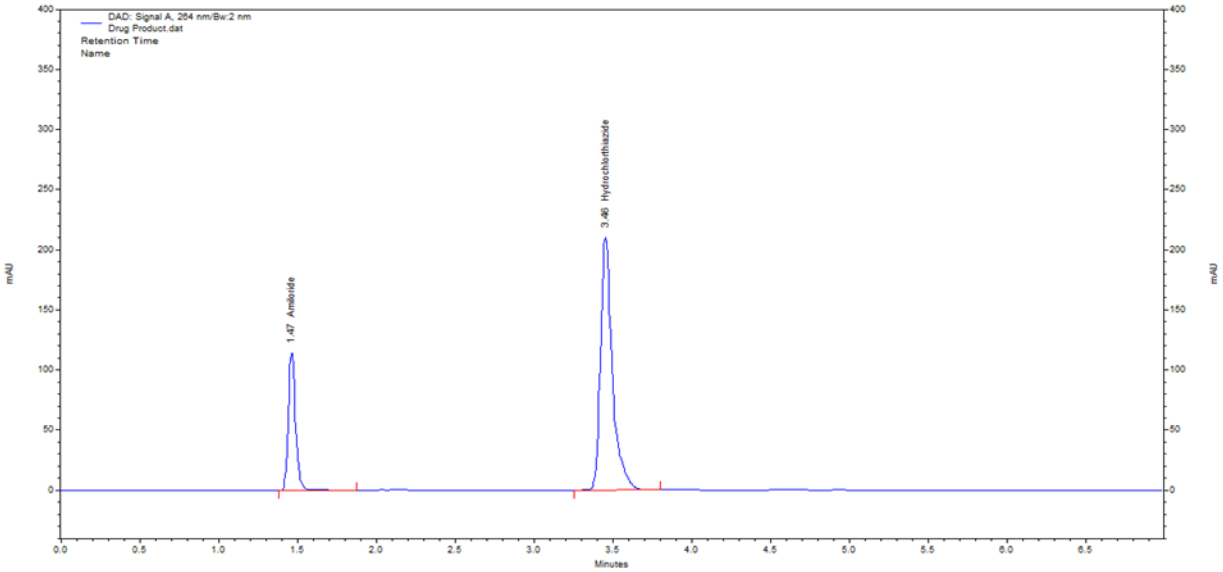

Figure no 8. Chromatogram of Amiloride and Hydrochlorothiazide in tablet formulation.

Amount of drug present in the marketed formulation was calculated using RP-HPLC. Amount of Amiloride and Hydrochlorothiazide was found to be 99.66 & 99.45 % respectively. This method can be employed for routine analysis of Amiloride and Hydrochlorothiazide. The result of assay of marketed formulation are given in Table 3.

The separation was achieved by Phenomenex C_8 column (150x4.6 mm, 5 um) column, at 1 ml/min flow rate, detection wavelength is 264 nm, mobile phase containing 0.1% Formic acid: Acetonitrile in ratio of 40:60.The detection was carried out at 264 nm. The retention time of Amiloride and Hydrochlorothiazide was found to be 1.47 ± 0.5 min and 3.47 ± 0.5 min respectively.

7.3) VALIDATION OF RP-HPLC METHODE:[36-41]

A. Linearity: Different concentration of solution prepared for Linearity of both Amiloride and Hydrochlorothiazide are shown in (Table 4 and Table -5) calibration curves are shown in Figure 9 & 10 respectively.

Table no.4 Linearity dilutions for Amiloride.

Amiloride		
% Level	Conc. (µg/ml)	Area
80	0.8	610589
90	0.9	682953
100	1	763696
110	1.1	834321
120	1.2	911264

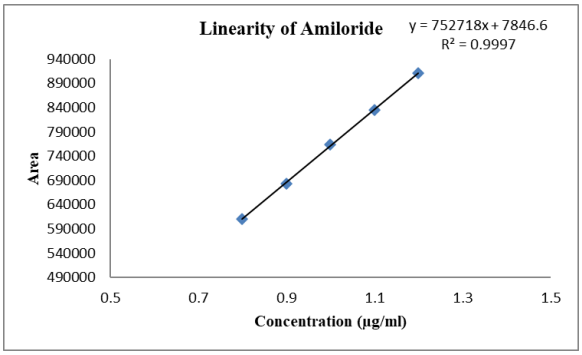

Figure no.9 calibration curve of Amiloride.

Table no.5 Linearity dilutions for Hydrochlorothiazide.

Hydrochlorothiazide		
% Level	Conc. (µg/ml)	Area
80	8	1853569
90	9	2071598
100	10	2307262
110	11	2530168
120	12	2765435

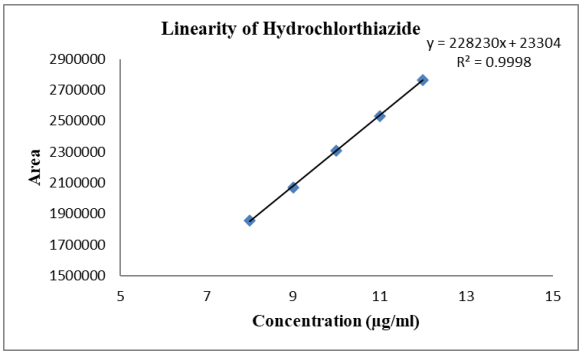

Figure no.10 Calibration curve of Hydrochlorothiazide.

According to ICH guideline linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of an analyte Linearity was studied by plotting a graph of area v/s concentration. A series standard solution of Amiloride and Hydrochlorothiazide were prepared in the concentration range of 0.8 µg/ml to 1.2 µg/mL and 8 µg/mL to 12 µg/mL respectively with linearity range 80-120% for both the drug and is shown in Table 7.10 and 7.11.

The regression coefficient (r^2) of Amiloride was found to be 0.999 & for Hydrochlorothiazide regression coefficient (r^2) was found to be 0.999. The equation of regression line for Amiloride was found to be y=75271x + 7846 for Hydrochlorothiazide was found to be y=22823x+23304 Linearity graph of Amiloride and Hydrochlorothiazide shown in figure 7.12 & 13 respectively

B. Precision:

The Precision study of Amiloride and Hydrochlorothiazide are shown Table 6 respectively.

Table no. 6 Precision of Amiloride and Hydrochlorothiazide.

Amiloride				
Condition	Sample ID	RT	Area	% Assay
Morning	WS	1.47	763696	-
	DP	1.47	761105	99.66
Evening	WS	1.47	760205	-
	DP	1.47	755548	99.39
% RSD				0.19
Day 2	WS	1.47	755075	-
	DP	1.47	749405	99.25
% RSD				0.21

Hydrochlorthiazide				
Condition	Sample ID	RT	Area	% Assay
Morning	WS	3.46	2307262	-
	DP	3.46	2294575	99.45
Evening	WS	3.46	2297504	-
	DP	3.46	2281759	99.31
% RSD				0.10
Day 2	WS	3.46	2293046	-
	DP	3.46	2275457	99.23
% RSD				0.11

The Precision of test results is ensured by intraday and interday precision. Amiloride and Hydrochlorothiazide both had % RSD values less than 2. Results are shown in Table 6.

C. Accuracy: The accuracy study of Amiloride and Hydrochlorothiazide are shown in Table 7 and 8 respectively.

Table no. 7. Accuracy Study of Amiloride.

Sample ID	Reps	Spiked Conc. (µg/ml)	Area	Amount Recovered (µg/ml)	% Recovery	AVG	STDEV	% RSD
80%	Rep 1	0.80	610589	0.80	99.96	100.06	0.13996	0.14
	Rep 2		612147	0.80	100.22			
	Rep 3		610758	0.80	99.99			
100%	Rep 1	1	763696	1.00	100.02	100.11	0.258408	0.26
	Rep 2		762869	1.00	99.91			

	Rep 3		766624	1.00	100.40			
120%	Rep 1	1.2	911264	1.19	99.46	99.54	0.074253	0.07
	Rep 2		912235	1.19	99.56			
	Rep 3		912575	1.20	99.60			

Table no. 8 Accuracy Study of Hydrochlorothiazide.

Sample ID	Reps	Spiked Conc. (µg/ml)	Area	Amount Recovered (µg/ml)	% Recovery	AVG	STDEV	% RSD
80%	Rep 1	8.00	1853569	8.05	100.58	100.08	0.446524	0.45
	Rep 2		1837598	7.98	99.71			
	Rep 3		1842140	8.00	99.96			
100%	Rep 1	10.00	2307262	10.02	100.15	99.94	0.252793	0.25
	Rep 2		2295874	9.97	99.66			
	Rep 3		2303684	10.00	100.00			
120%	Rep 1	12.00	2765435	12.00	100.04	99.82	0.206803	0.21
	Rep 2		2754014	11.95	99.62			
	Rep 3		2759254	11.98	99.81			

The method's accuracy defines how close the method's results are to the true value. The results of the accuracy testing revealed that the technique is accurate within acceptable ranges. When the % RSD for Amiloride and Hydrochlorothiazide is calculated, all of the results are within acceptable bounds. A maximum RSD of 2.0% indicated acceptable accuracy within the range. The results are shown in Table 7 and 8.

According to the Accuracy research, the percent recovery of Amiloride is 99.54-100.11 % and Hydrochlorothiazide is 99.82-100.08 %, both of which are within the ICH standards.

D. Limit of Detection (LOD) and Limit of Quantification (LOQ):

The LOD and LOQ of Amiloride and Hydrochlorothiazide are shown in Table 7.18.

Table no.9 The LOD and LOQ of Amiloride and Hydrochlorothiazide.

Sr.No	Name of drug	LOD (µg/mL)	LOQ (µg/mL)
1.	Amiloride	0.03	0.10
2.	Hydrochlorothiazide	0.24	0.72

Sensitivity of the method was determined with respect to limit of detection (LOD) and the quantification limit of an individual analytical procedure is the lowest amount of an analyte in a sample which can be quantitatively determined with the suitable precision and accuracy.

LOD and LOQ value for Amiloride was found to be 0.03 µg/mL and 0.10 µg/mL, LOD and LOQ value for Hydrochlorothiazide was found to be 0.24 µg/mL and 0.72 µg/mL respectively. Results are shown in Table 9.

E. System suitability:

System suitability data of Amiloride and Hydrochlorothiazide given in below Table 10.

Table no. 10 System suitability parameter of Amiloride.

Amiloride					
Sample ID	Area	RT	TP	Asymmetry	Resolution
100% Rep 1	763696	1.47	4948	1.32	0.00
100% Rep 2	762869	1.47	5045	1.30	0.00
100% Rep 3	766624	1.47	4822	1.28	0.00
100% Rep 4	763245	1.47	4917	1.31	0.00
100% Rep 5	762541	1.47	4866	1.29	0.00
100% Rep 6	762226	1.47	4937	1.33	0.00
AVG	763534	1.47			
STDEV	1599.813	0			
% RSD	0.21	0.00			

Table no. 11 System suitability parameter of Hydrochlorothiazide.

Hydrochlorthiazide					
Sample ID	Area	RT	TP	Asymmetry	Resolution
100% Rep 1	2307262	3.46	11887	1.53	18.95
100% Rep 2	2295874	3.46	12104	1.54	18.95
100% Rep 3	2303684	3.46	11752	1.51	18.95
100% Rep 4	2319952	3.46	12057	1.52	18.95
100% Rep 5	2303175	3.46	11368	1.55	18.95
100% Rep 6	2292254	3.46	12355	1.52	18.95
AVG	2303700	3.46			
STDEV	9688.637	4.86E-16			
% RSD	0.42	0.00			

The system, method, and column performance were validated by testing system suitability features. Six times, a standard solution of Amiloride and Hydrochlorothiazide was injected into the system, and the system's suitable features were evaluated. Results are shown in Table 10 and 11.

F. Robustness: Robustness data of Amiloride and Hydrochlorothiazide given in below

Table no.12 Robustness parameter of Amiloride and Hydrochlorothiazide.

Variation in Column temperature (Amiloride)							
Condition	Sample ID	RT	Area	% Assay	Average	STDEV	% RSD
28°C	WS	1.47	761145	-	99.60	0.067594	0.07
	DP	1.47	757540	99.53			
30°C	WS	1.47	763696	-			
	DP	1.47	761105	99.66			
32°C	WS	1.47	762140	-			
	DP	1.47	759141	99.61			

Variation in wavelength (Amiloride)							
Condition	Sample ID	RT	Area	% Assay	Average	STDEV	% RSD
262 nm	WS	1.47	760256	-	99.60	0.058838	0.06
	DP	1.47	757054	99.58			
264 nm	WS	1.47	763696	-			
	DP	1.47	761105	99.66			
266 nm	WS	1.47	771947	-			
	DP	1.47	768447	99.55			

Variation in Column temperature (Hydrochlorthiazide)							
Condition	Sample ID	RT	Area	% Assay	Average	STDEV	% RSD
28°C	WS	3.46	2294754	-	99.40	0.044033	0.04
	DP	3.46	2280488	99.38			
30°C	WS	3.46	2307262	-			
	DP	3.46	2294575	99.45			
32°C	WS	3.46	2297874	-			

	DP	3.46	2283399	99.37			

Variation in Column temperature (Hydrochlorthiazide)							
Condition	Sample ID	RT	Area	% Assay	Average	STDEV	% RSD
262 nm	WS	3.46	2282545	-	99.46	0.034142	0.03
	DP	3.46	2271133	99.50			
264 nm	WS	3.46	2307262	-			
	DP	3.46	2294575	99.45			
266 nm	WS	3.46	2321848	-			
	DP	3.46	2308723	99.43			

Robustness was investigated using various deliberate alterations in chromatographic settings, such as changes in column Condition like 28˚C, 30˚C and 32˚C.

RSD was shown to be less than 2% in the Amiloride and Hydrochlorothiazide robustness studies. As a result, it is strong and adheres to ICH criteria. Results are shown in Table 12.

CONCLUSION

Amiloride and hydrochlorothiazide may be easily and quickly quantified from their formulations using the RP-HPLC method that has been developed and validated. Every validation parameter was determined to be within the permissible ranges in compliance with ICH criteria. Regardless of the excipients present, the proposed method was found to be straightforward, accurate, exact, robust, and resilient. It was also shown to be specific for the pharmaceuticals of interest. It can be applied to the regular examination of commercially available formulations.

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