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New Validated Stablity Indicating Rp-Hplc Bioanalytical Method Development and Validation for Simultaneous Estimation of Hydrochlorothiazide, Ramipril and Losartan in Human Plasma by Using PDA Detector

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Abstract

A precised and reproducible stability indicating RP-HPLC method was developed for the simultaneous estimation of hydrochlorothiazide, ramipril and losartan potassium in plasma by using Symmetry C_{18} column (4.6 x 150mm, 5m, Make: Hypersil) in an isocratic mode. The drug was spiked in the plasma and extracted with mobile phase by protein precipitation method. The mobile phase was consisted of potassium dihydrogen phosphate (KH_2PO_4) and acetonitrile [HPLC Grade] in the ratio of 68:32 (% v/v). The detection was carried out at 210 nm. The percentage mean recoveries of hydrochlorothiazide, ramipril and losartan potassium were found to be 98.21-101.13, 98.82-100.93 and 99.69-100.98 percentage respectively. This reveals that the method is quite accurate. The method was linear over the concentration range for hydrochlorothiazide 12.5-32.5, ramipril 1.25-3.25 and losartan 50.0 -130.0 µg/mL. The percentage relative standard deviation for inter-day and intra-day precision was found to be within limits. The lower limit of quantification was found to be 0.647, 1.283 and 2.647 µg/mL for hydrochlorothiazide, ramipril and losartan respectively. The percentage relative standard deviation obtained for the drugs spiked in plasma for stability studies were less than 2 %. The validation of method was carried out utilizing ICH-guidelines.

Keywords: Losartan potassium; Ramipril; Hydrochlorothiazide; Validation; RP-HPLC; Bioanalytical method

Introduction

Hydrochlorothiazide (HCT), chemically described as 6-Chloro-3, 4-dihydro-2H-1, 2, 4-benzothiadiazine-7- sulfonamide 1, 1-dioxide which is used as a diuretic [1]. In the chemical analysis of multicomponent dosage form one drug may interfere with estimation of other drug [2]. Ramipril (RAM) is chemically describe as (2*S*, 3*aS*, 6*aS*)-1[(*S*)-*N*-[(*S*)-1-carboxy-3 phenylpropyl] alanyl] octahydrocyclopenta [b] pyrrole-2-carboxylic acid-1-ethyl ester. It is an antihypertensive agent. Ramiprilat, the diacid metabolite of Ramipril, has a non-sulfhydryl angiotensin converting enzyme inhibitor [3,4]. RAM was converted to Ramiprilat by hepatic cleavage of the ester group. Losartan potassium (LOS) is chemically described as [4(2hydroxy3isopropylaminopropoxy) phenylacetamide] and is a competitive antagonist and inverse agonist of A-II [5,6]. Hence analytical methods were developed to estimate all the drugs simultaneously in multicomponent formulations. Many analytical methods like UV Spectrophotometric, HPTLC, electrochemical, radioimmunoassay were reported for determination of LOS and HCT in alone and in combination with other antihypertensive drugs. The RP-HPLC method was reported for simultaneous estimation of LOS and HCT [7-15].

A literature survey regarding quantitative analysis of these drugs revealed that a few methods were reported for the estimation of LOS and RAM individually and only some method is reported so far for the estimation of LOS and RAM in combined dosage forms.

However no one has reported RP-HPLC method for the simultaneous estimation of all these drugs together till dated in plasma. In this communication we reported a RP-HPLC method for the development as well as the validation for the simultaneous estimation of hydrochlorothiazide, ramipril and losartan in plasma. The chemical structure of hydrochlorothiazide, ramipril and losartan are represented in Figures 1, 2 and 3 respectively.

Materials and Method [16,17]

Chemicals and reagents used

The reference samples of hydrochlorothiazide, ramipril and losartan potassium were supplied by M/s Pharma Train, Hyderabad. HPLC grade water (prepared by using 0.45 Millipore Milli-Q) was procured from Standard Reagents, Hyderabad. HPLC grade methanol was purchased from Merck, Mumbai. The chemicals used for preparation of buffer include potassium dihydrogen phosphate (Finar Chemicals, Ahmedabad), and orthophosphoric acid (Standard Reagents, Hyderabad). The processed plasma was collected from M/s. Pharma Train, Hyderabad, Andhra Pradesh.

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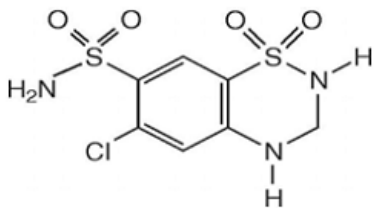

Figure 1: Chemical structure of Hydrochlorothiazide.

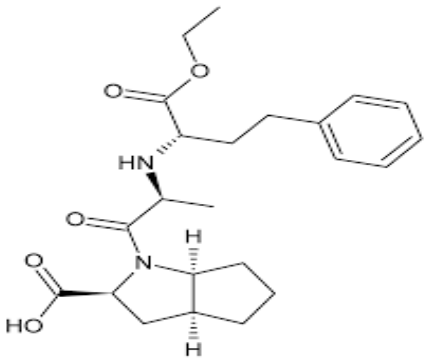

Figure 2: Chemical structure of Ramipril.

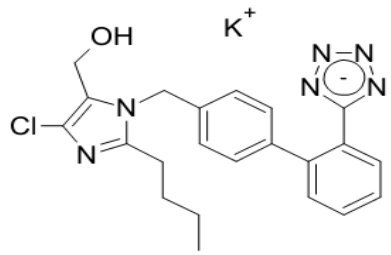

Figure 3: Chemical structure of losartan potassium.

0.45 µ membrane filters (Advanced Micro Devices Pvt. Ltd., Chandigarh, India) were used for filtration of various solvents and solutions intended for injection into the column.

Instrumentation

A Waters Alliance 2695 separation module equipped with a 2487 UV detector was employed throughout this study. Column that was employed in the method was Symmetry C_{18} column (4.6 x 150 mm, 5 µm, Make: Hypersil). The samples were injected with an automatic injector. The 20 L volume of sample was injected. The input and output operations of the chromatographic system were monitored by Waters Empower software. The flow rate selected was 0.9 mL per min. The detection was done at 210 nm. The temperature and run time was monitored at 25°C and 14.0 min respectively.

The ultra violet spectra of the drugs used for the investigation were taken on a Lab India UV 3000 spectrophotometer for finding out their λ_{max} values.

Solubility of the compounds was enhanced by sonication on an ultra sonicator (Model: Power Sonic 510, Hwashin Technology).

All the weighings in the experiments were done with an Afcoset electronic balance. The HermLe microlitre centrifuge Z100 (Model no. 292 P01) was used for the centrifugation process and Remi equipments (Model no. CM101DX) Cyclomixer was used.

Glassware

All the volumetric glassware used in the study was of Grade A quality Borosil.

Preparation of phosphate buffer [18]

The buffer solution was prepared by dissolving 7.0 grams of potassium dihydrogen phosphate in 900 mL of HPLC grade water in a 1000 mL clean and dry flask. The mixture was stirred well until complete dissolution of the salt. The volume was made upto the mark with water. The pH was adjusted to 4.0 with 1 % ortho phosphoric acid.

Preparation of mobile phase

The mobile phase was prepared by mixing 680 mL of phosphate buffer (pH 4.0) and 320 mL of acetonitrile HPLC in a 1000 mL clean and dry flask. The resultant mobile phase was filtered through a 0.45 µ membrane filter (Advanced Micro Devices Pvt. Ltd., Chandigarh, India) under vacuum. The resultant mobile phase was degassed in an ultra sonicator for 5 min.

Diluent preparation

The same mobile phase was used as diluent. This solution was used to dilute the drug solutions in the study.

Preparation of standard solution of hydrochlorothiazide, ramipril and losartan

10 mg hydrochlorothiazide was weighed accurately and transferred into a 100 mL clean and dry volumetric flask. Initially the drug was dissolved with 70 mL of diluent. The solution was sonicated for 15 min. to dissolve the drug completely. The final volume was made up to the mark with the same solvent. Similarly, 1 mg ramipril was weighed accurately and transferred into a 100 mL clean and dry volumetric flask. Initially the drug was dissolved with 70 mL of diluent. The solution was sonicated for 15 min. to dissolve the drug completely. The final volume was made up to the mark with the same solvent. Similarly, 10 mg of losartan was weighed accurately and transferred into a 10 mL clean and dry volumetric flask. Initially the drug was dissolved with 7 mL of diluent. The solution was sonicated for 15 min. to dissolve the drug completely. The final volume was made up to the mark with the same solvent.

From the above prepared solutions 2.25 mL of hydrochlorothiazide, 2.25 mL of ramipril and 0.9 mL of losartan was pipetted out into a 10 mL clean and dry volumetric flask and it was diluted up to the mark with the same diluent. This mixed stock solution contains 22.5 µg/mL of hydrochlorothiazide, 2.25 µg/mL of ramipril and 90.0 µg/mL of losartan.

Spiking of Hydrochlorothiazide, Ramipril and Losartan into plasma and their extraction from plasma (By protein precipitation method)

From the above prepared mixed stock (22.5 µg/mL of hydrochlorothiazide, 2.25 µg/mL of ramipril and 90.0 µg/mL of losartan), 0.5 mL was pipetted out and spiked into 0.5 mL of plasma in a polypropylene tube (Torson's). Then the tube was cyclo mixed for 5 min. Then 1.0 mL of acetonitrile was added to the tube and centrifuged

for 20 min at 3000 rpm. The supernatant liquid was collected in another Eppendorf tube and 20 µL supernatant was injected into the analytical column.

Validation Development [19,20]

Selectivity

An aqueous mixture of hydrochlorothiazide, ramipril and losartan [22.5 µg/mL, 2.25 µg/mL and 90.0 µg/mL concentration] was prepared and injected in the column and the retention times were checked and any interference at the retention times were checked by comparing the response in the blank. There were no interferences found in the retention times of the drugs extracted from plasma. Hence, the method was found to be precised and specific. A typical chromatogram of Hydrochlorothiazide, Ramipril and Losartan in plasma is shown in Figure 4.

Sensitivity

To determine the sensitivity in terms of LLOQ, 'Lower Limit of Quantification' where the response of LLOQ must be at least five times greater than the response of interference in blank matrix at the retention time of the analyte(s). The LLOQ obtained by the proposed method for hydrochlorothiazide, ramipril and losartan were 0.647, 1.283 and 2.647 µg/mL respectively.

Precision

To check the intra and inter-day variations of the method, solutions containing 22.5 µg/mL of hydrochlorothiazide, 2.25 µg/mL of ramipril and 90.0 µg/mL of losartan were subjected to the proposed HPLC method of analysis and results obtained were noted. The precision of the proposed method i.e. the intra and inter-day variations in the peak areas of the drugs solutions in plasma were calculated in terms of percent relative standard deviation and the results are presented in Tables 1-6. A statistical evaluation revealed that the relative standard deviation of the drugs at linearity level for 6 injections was less than 2.0. Typical chromatogram of metformin and alogliptin in plasma for intra and inter-day precision are shown in Figure 5 and 6.

Accuracy

To determine the accuracy of the proposed method, recovery studies were carried out by analyzing (8.0, 10.0, 12.0 mg of hydrochlorothiazide, ramipril and losartan) of pure drugs. The drugs solutions were diluted at linearity level (22.5 µg/mL of hydrochlorothiazide, 2.25 µg/mL of ramipril and 90.0 µg/mL of losartan). Then each dilution was injected thrice (n=3). The percent recoveries of the drugs were determined. The results are shown in Table 7, 8 and 9.

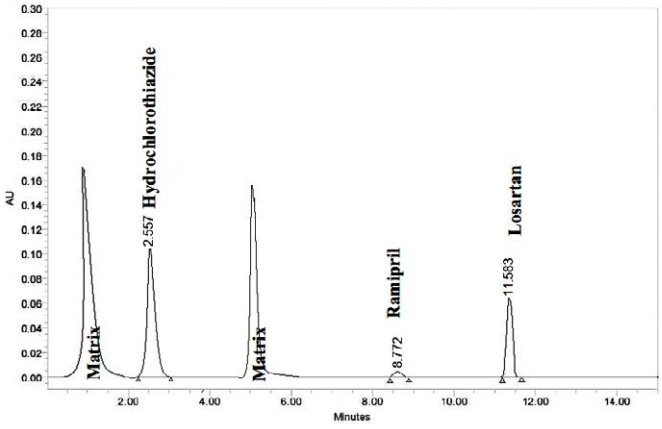

Figure 4: A typical chromatogram of Hydrochlorothiazide, Ramipril and Losartan standard drugs in plasma.

Injection	Retention Time	Peak area
Injection-1	2.558	180230
Injection-2	2.557	181075
Injection-3	2.556	181910
Injection-4	2.557	181530
Injection-5	2.556	182350
Injection-6	2.558	181075
Average	2.557	181419.0
Standard Deviation	0.009	814.3
% RSD	0.02	0.5

Table 1: Intra-day precision of the proposed method for Hydrochlorothiazide in plasma.

Injection	Retention Time	Peak area
Injection-1	8.775	69076
Injection-2	8.774	71040
Injection-3	8.773	70669
Injection-4	8.772	69784
Injection-5	8.774	70605
Injection-6	8.773	71040
Average	8.773	70235
Standard Deviation	0.001	794
% RSD	0.11	1.1

Table 2: Intra-day precision of the proposed method for Ramipril in plasma.

Injection	Retention Time	Peak area
Injection-1	11.582	572114
Injection-2	11.582	580970
Injection-3	11.583	587248
Injection-4	11.582	596978
Injection-5	11.585	588160
Injection-6	11.584	588097
Average	11.583	585594.5
Standard Deviation	0.001	8345.92
% RSD	0.01	1.42

Table 3: Intra-day precision of the proposed method for Losartan in plasma.

Days	Retention Time	Peak area
Day-1*	2.557	180230
Day -2*	2.557	181075
Day -3*	2.554	181910
Average	2.557	181071
Standard Deviation	0.002	840.0
% RSD	0.06	0.46

Table 4: Inter-day precision of the proposed method for Hydrochlorothiazide in plasma (on three consecutive days n = 6).

Linearity

In order to find out the linearity range of the proposed HPLC method in plasma, curves were constructed by plotting peak areas obtained for the analyte against their concentrations. A good linear relationship (r^2=0.999) was observed between the concentrations of hydrochlorothiazide, ramipril and losartan and their corresponding

Days	Retention Time	Peak area
Day -1*	8.774	71316
Day -2*	8.774	71804
Day -3*	8.773	71774
Average	8.773	71631
Standard Deviation	0.006	273.5
% RSD	0.007	0.38

Table 5: Inter-day precision of the proposed method for Ramipril in plasma (on three consecutive days n = 6).

Days	Retention Time	Peak area
Day-1*	11.582	572114
Day-2*	11.580	578110
Day-3*	11.582	579181
Average	11.581	576468
Standard Deviation	0.001	3809
%RSD	0.01	0.66

*Average of Six injections

Table 6: Inter-day precision of the proposed method for Losartan in plasma (on three consecutive days n = 6)

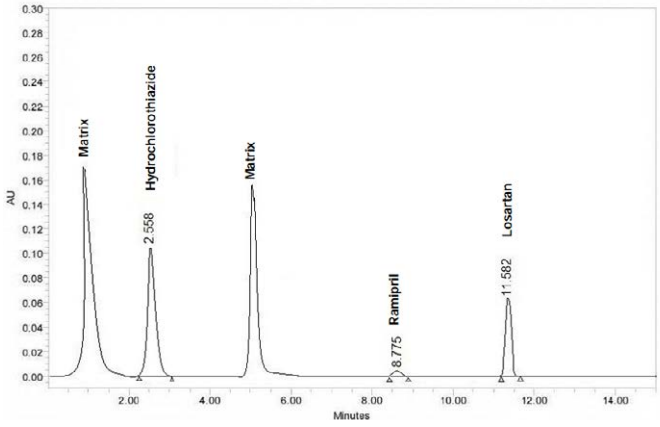

Figure 5: Typical chromatogram of hydrochlorothiazide, ramipril and losartan in plasma for intra-day precision.

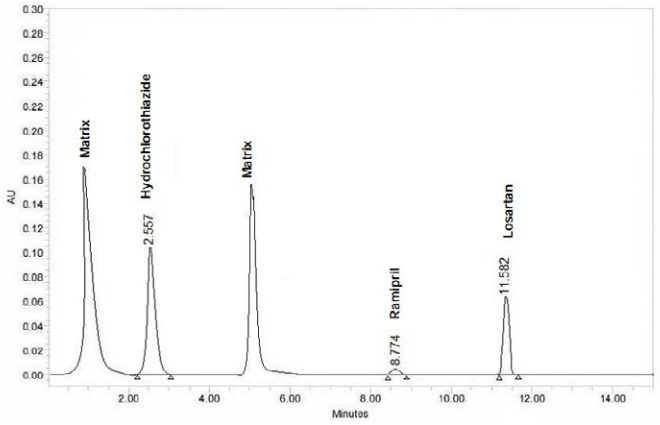

Figure 6: Typical chromatogram of hydrochlorothiazide, ramipril and losartan in plasma for inter-day precision.

peak areas. The relevant regression equations were y = 8339x – 2561 for hydrochlorothiazide (r^2=0.999), y = 30849x + 2946 for ramipril (r^2=0.998) and y = 6369x + 33103 for losartan (r^2=0.999) (where y is the peak area and x is the concentrations of hydrochlorothiazide, ramipril and losartan (µg/mL)). The slope, intercept and the correlation coefficient of the plots are shown in Tables 10-12. The linearity ranges for hydrochlorothiazide, ramipril and losartan and their corresponding graphs are shown in Figures 7-9.

Stability

All stability determinations used a set of samples prepared from

Conc. Level	% Recovery	Avg. % Recovery	Amount Recovered (mg)	SD	% RSD
80 %	98.26	98.21	7.86	0.006	0.07
	98.29		7.86		
	98.07		7.85		
100 %	99.63	99.80	9.96	0.017	0.17
	99.88		9.99		
	99.88		9.99		
120 %	100.92	101.13	12.11	0.031	0.25
	101.07		12.13		
	101.39		12.17		

Table 7: Accuracy data of the proposed method for Hydrochlorothiazide in plasma.

Conc. Level	% Recovery	Avg. % Recovery	Amount Recovered (mg)	SD	% RSD
80 %	99.21	98.82	7.94	0.04	0.44
	98.89		7.91		
	98.36		7.87		
100 %	100.38	99.80	10.04	0.05	0.55
	99.77		9.98		
	99.25		9.93		
120 %	101.65	100.93	12.2	0.075	0.63
	100.64		12.08		
			12.06		

Table 8: Accuracy data of the proposed method for Ramipril in plasma.

Conc. Level	% Recovery	Avg. % Recovery	Amount Recovered (mg)	SD	% RSD
80 %	99.70	99.69	7.98	0.015	0.19
	99.88		7.99		
	99.5		7.96		
100 %	99.38	99.80	9.94	0.040	0.40
	99.83		9.98		
	100.19		10.02		
120 %	100.77	100.98	12.09	0.046	0.38
	100.73		12.09		
	101.44		12.17		

Table 9: Accuracy data of the proposed method for Losartan in plasma.

Concentration (µg/mL)	Area	Statistical Analysis
12.5	101321	Slope= 8339 Intercept= -2561 C. C= 0.999
17.5	145664	
22.5	182546	
27.5	226332	
32.5	269461	

Table 10: Linearity range of Hydrochlorothiazide in plasma.

Concentration (µg/mL)	Area	Statistical Analysis
1.25	41903	Slope= 30849 Intercept= 2946 C. C= 0.998
1.75	55651	
2.25	72903	
2.75	88951	
3.25	102376	

Table 11: Linearity range of Ramipril in plasma.

Concentration (µg/mL)	Area	Statistical Analysis
50	348203	Slope= 6369 Intercept= 33103 C. C= 0.999
70	481790	
90	606289	
110	738597	
130	856704	

Table 12: Linearity range of Losartan in plasma.

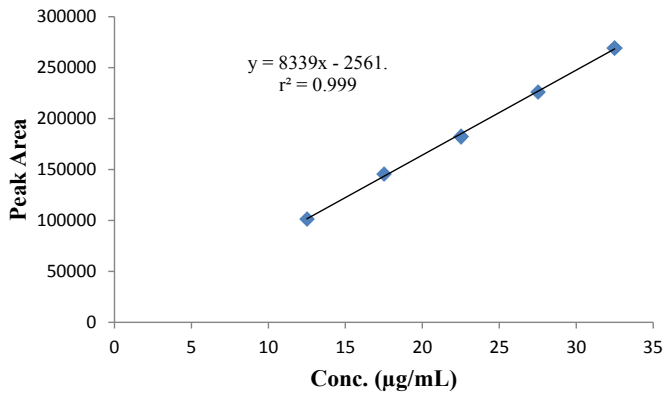

Figure 7: Calibration curve for Hydrochlorothiazide in plasma.

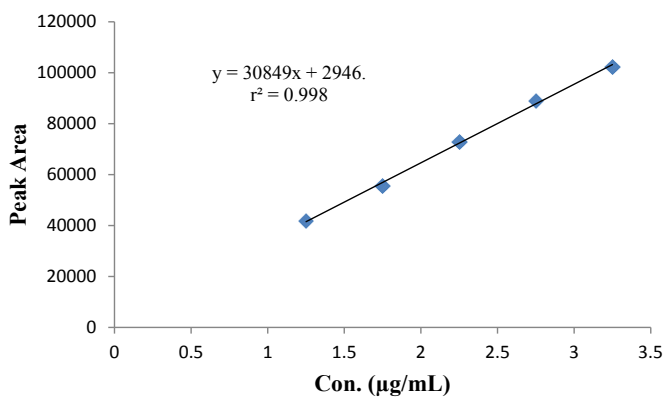

Figure 8: Calibration curve for Ramipril in plasma.

a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free biological matrix. The stock solutions of the analyte for stability evaluation were prepared in an appropriate solvent at known concentrations. To test the stability of the drug extract, it was subjected to

a) Freeze and thaw stability at -20°C ± 2°C,

b) Short term stability for period of 24 hours stored at room temperature,

c) Long term stability for period of 15 days stored at 4°C.

Similar to the preparation of the standard preparation, the above samples were spiked into the plasma and extracted and collected in vial and injected into HPLC system. All the stability samples compared against the standard stock solution assessed for stability. The results are presented in Tables 13-15 (the figures in the table are in peak area units). Typical chromatograms for standard samples, freeze and thaw stability samples, short term stability samples and long term stability samples were represented in Figures 10-13.

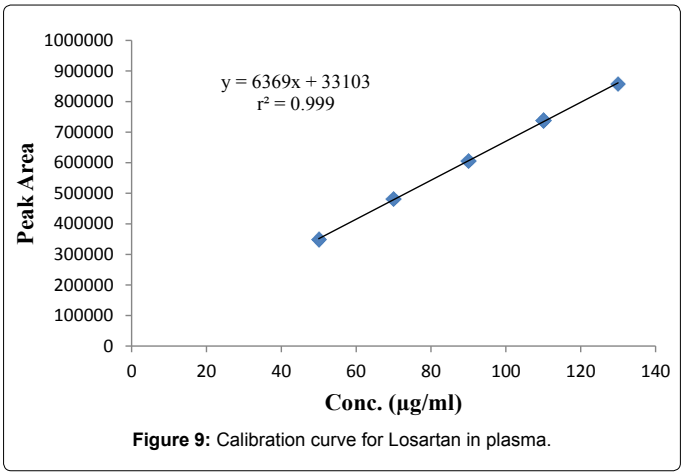

Figure 9: Calibration curve for Losartan in plasma.

Sr. No.	Standard Sample	Freeze and Thaw Stability Sample	Short term Stability Sample	Long term Stability Sample
1	182544	179258	174877	171628
2	182338	179022	174865	171452
3	182746	179365	174822	171821
Mean	182543	179215	174855	171634
SD	204	176	29	185
% RSD	0.11	0.10	0.02	0.11
Assay	--------	98.18	95.79	94.02

Table 13: The Stability data for Hydrochlorothiazide in plasma.

Sr. No.	Standard Sample	Freeze and Thaw Stability Sample	Short term Stability Sample	Long term Stability Sample
1	72901	71296	69162	68513
2	72740	71169	69060	68482
3	73102	71374	69263	68604
Mean	72914	71280	69162	68533
SD	181	103	102	63
% RSD	0.25	0.12	0.15	0.09
Assay	---------	97.76	94.61	93.99

Table 14: The Stability data for Ramipril in plasma.

Sr. No.	Standard Sample	Freeze and Thaw Stability Sample	Short term Stability Sample	Long term Stability Sample
1	606285	598221	572212	559946
2	606360	598214	572014	559964
3	606199	598305	573247	559291
Mean	606281	598247	572491	559734
SD	81	51	662	383
% RSD	0.01	0.01	0.12	0.07
Assay	--------	98.67	94.43	92.31

Table 15: The Stability data for Losartan in plasma.

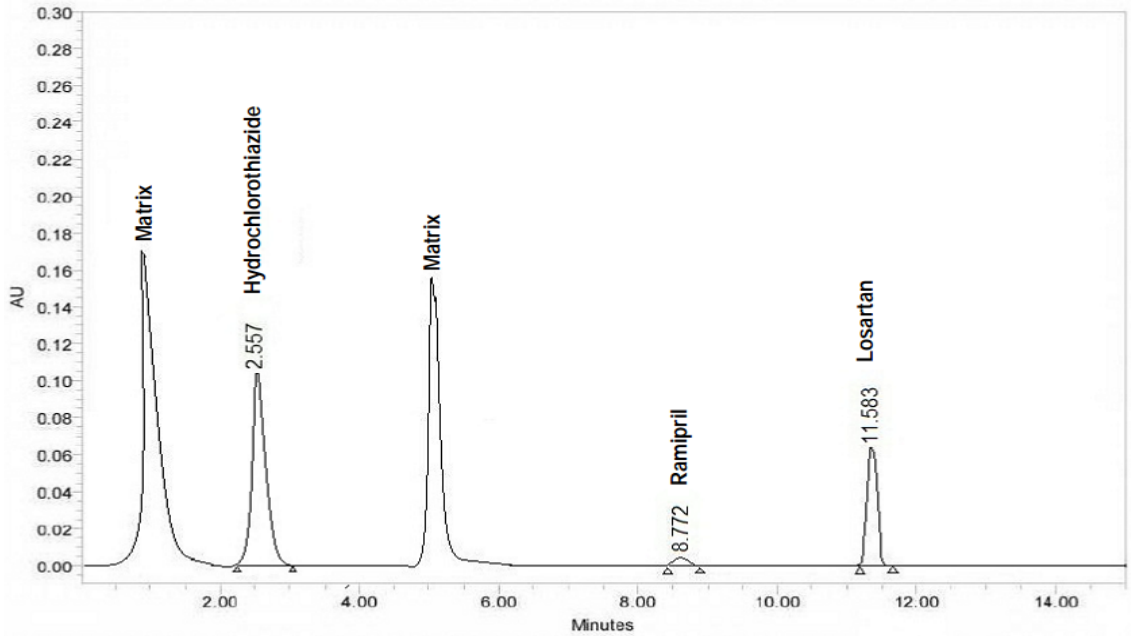

Figure 10: Typical chromatogram for standard samples of hydrochlorothiazide, ramipril and losartan in plasma for stability studies.

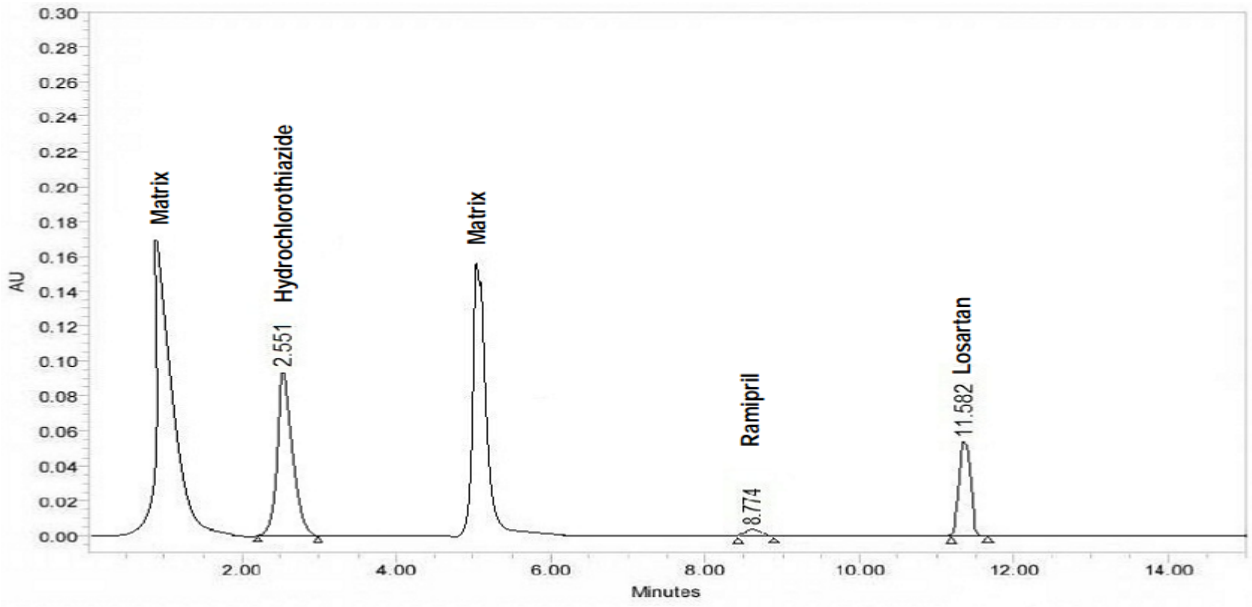

Figure 11: Typical chromatogram for freeze thaw samples of hydrochlorothiazide, ramipril and losartan in plasma for stability studies.

Results and Discussion

To optimize the mobile phase, various proportions of water with methanol (HPLC grade) were tested. The use of water and methanol (HPLC grade) in the ratio of 68:32 (v/v) resulted in peak with good shapes and resolution. A flow rate of 0.9 mL /min was found to be optimum in the 0.4-1.5 mL/min range resulting in short retention time, baseline stability and minimum noise.

The LLOQ obtained for hydrochlorothiazide, ramipril and losartan by the proposed method in plasma was 0.647, 1.283 and 2.647 µg/mL respectively. The retention times obtained for hydrochlorothiazide, ramipril and losartan in plasma were observed at 2.557, 8.774 and 11.582 min respectively. Quantitative linearity of drug in plasma was obeyed in the concentration ranges of 12.5-32.5 µg/mL for hydrochlorothiazide, 1.25-3.25 µg/mL for ramipril and 50-130 µg/mL for losartan, respectively. The relevant regression equations were y = 8339x – 2561 for hydrochlorothiazide, y = 30849x + 2946 for ramipril and y = 6369x + 33103 for losartan (where y is the peak area and x is the concentration of hydrochlorothiazide, ramipril and losartan (µg/mL)). The intra-day and inter-day drugs variations in plasma by the

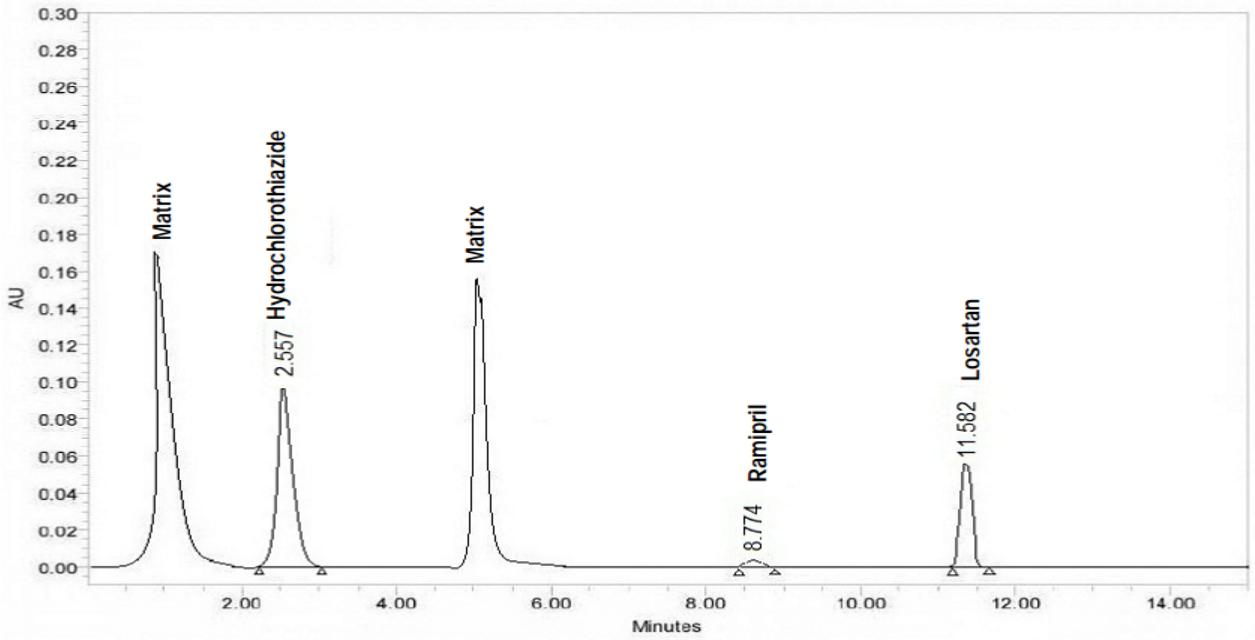

Figure 12: Typical chromatogram for short term stability samples of hydrochlorothiazide, ramipril and losartan in plasma for stability studies.

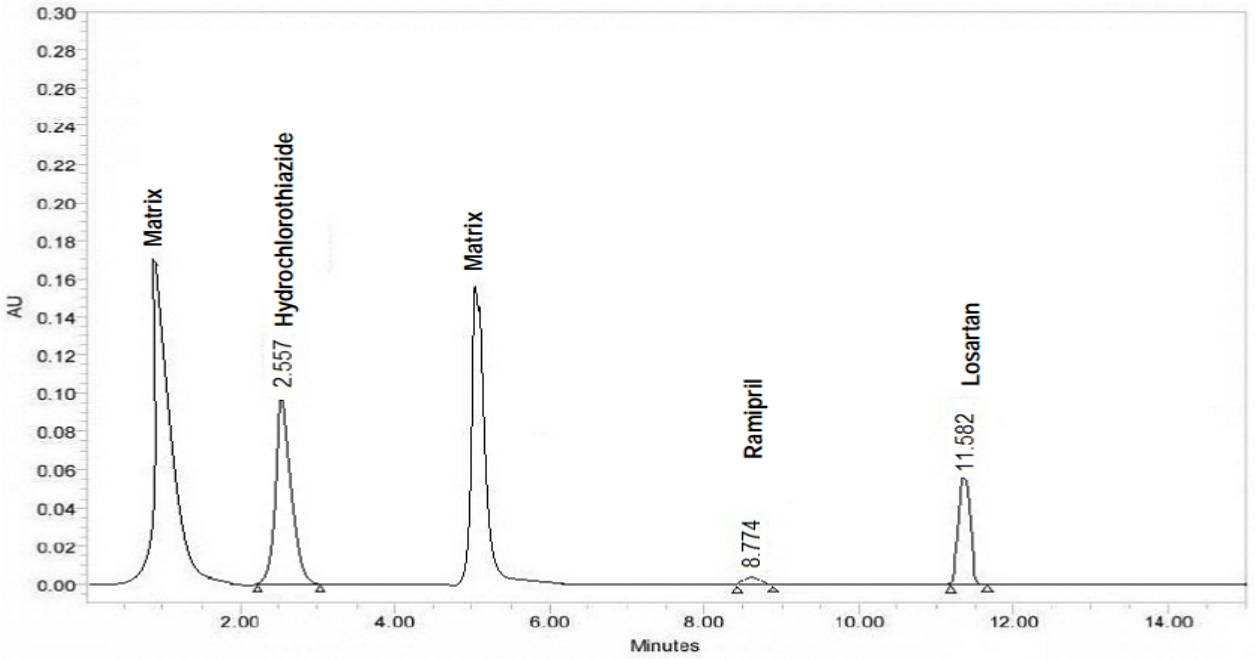

Figure 13: Typical chromatogram for long term stability samples of hydrochlorothiazide, ramipril and losartan in plasma for stability studies.

proposed method showed an RSD less than 2 %, indicating that the method is precise. The corresponding mean recoveries of the drugs in plasma were 98.21- 100.98 %. This reveals that the method is quite accurate. The RSD obtained for the drugs spiked in plasma for stability studies were less than 2 %.

Conclusion

The proposed HPLC method was found to be simple, precise, accurate and sensitive for the simultaneous determination of hydrochlorothiazide, ramipril and losartan. The method was validated as per ICH guidelines and all the parameters met the required acceptance criteria. Applicability of this method for simultaneous estimation of losartan, ramipril and hydrochlorothiazide in plasma was confirmed.

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for providing a reference sample of hydrochlorothiazide, ramipril and losartan and processed plasma.

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