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A Novel Stability Indicating RP-HPLC Method Development and Validation for The Simultaneous Estimation of Losartan Potassium, Ramipril and Hydrochlorothiazide in Bulk and Pharmaceutical Dosage Form

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A novel stability indicating reverse phase liquid chromatographic method has been developed for the simultaneous estimation of Losartan Potassium, Ramipril and Hydrochlorothiazide in bulk and Pharmaceutical formulations by using reverse phase Kromasil 100-5 C_{18} column [250mm x 4.6mm].The mobile phase (Methanol: phosphate buffer-pH 4.0) in the ratio of 70:30% v/v was pumped at a flow rate of 0.8ml/min and the column effluents were monitored at 233nm using Variable Wavelength UV detector. Linearity was obtained in the concentration range of 100-500 µg/ml for Losartan Potassium, 10-50 µg/ml for Ramipril and 20-100µg/ml for Hydrochlorothiazide. The established method was statistically validated according to the ICH Q2B guidelines and the percentage relative standard deviation for precision, robustness and ruggedness was found to be less than 2% indicating high degree of precision and robustness. The percentage recovery for the accuracy was found to be 100.76%, 96% and 98% for Losartan Potassium, Ramipril, and Hydrochlorothiazide respectively which were within the specified limits of recovery. Assay for the marketed formulation proved that 100.76% of Losartan Potassium and 96% of Ramipril and 98.22% of Hydrochlorothiazide. The proposed analytical method was observed for various stress condition and it was proved that more stable even under acidic, alkaline, peroxide and

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thermal degradation conditions. Hence due to its simplicity, rapidity, precision, accuracy and stability the developed HPLC method can be applied for the estimation of Losartan Potassium, Ramipril and Hydrochlorothiazide in pure and marketed formulations by a modern analyst.

Keywords: Losartan Potassium, Ramipril, Hydrochlorothiazide, RP-HPLC, Stability and Method validation

INTRODUCTION

Losartan potassium (LOS) is chemically described as 2-butyl-4-chloro-1{[2'-(1H-tetrazol-5-yl)][1,1'-biphenyl]-4-yl]methyl}-1H-imidazol (Fig.1.a) and is mainly used to treat high blood pressure(hypertension) as it is a competitive antagonist and inverse agonist of Angiotensin-II receptor. Ramipril (RAM) is (2S, 3aS,6aS) – 1 - [(2S) – 2 - {[(2S)-1-ethoxy-1-oxo-4phenylbutayl] amino} propanoyl] – octahydrocyclopenta [b] pyrrole-2-carboxylic acid (Fig.1.b). Used to treat hypertension and congestive heart failure. It is a angiotensin converting enzyme inhibitor (ACE). Hydrochlorothiazide (HCTZ) is 6-chloro-3, 4-dihydro-2H-1,2,4-benzothiadiazin sulfonamide 1,1-dioxide (Fig.1.c) used as diuretic [1-4].

Extensive literature search revealed that only very few methods are reported for the estimation of Losartan Potassium, Ramipril and Hydrchlorothiazide in combined dosage forms [5-14]. To the best of knowledge, no method has been reported for the simultaneous determination of three drugs in tablet dosage form. The objective of the present work was to design a validation procedure which can determine the three drugs in tablet dosage form with economical and ecofriendly mobile phase, with good resolution and peak symmetry. International conference on harmonization (ICH) has made mandatory the need of developing stability indicating assay methods for every drug candidate. There stability indicating assay methods helps in establishing the inherent stability of the drug which provides assurance on detection changes in identity, purity and potency of the product on exposure to various conditions. In the present study the drugs were exposed to a variety of stress like acidic, caustic, thermal, photolytic and oxidative stress conditions [15-16]. According to ICH guidelines the stress testing of the drug substances helps in identifying the likely degradation products, which in turn can help in establishing the degradation pathways and the intrinsic stability of the molecule.

MATERIALS AND METHODS

Equipment used

The chromatographic separation was performed on Agilent 1120 compact liquid chromatographic system integrated with a variable wavelength programmable UV detector and a Rheodyne injector equipped with 20µl fixed loop. A reverse phase Kromasil 100-5 C_{18} column [250mm x 4.6mm] was used. Lab India 3000+double beam UV visible spectrophotometer and Axis AGN204-PO electronic balance was used for spectrophotometric determinations and weighing purposes respectively.

Reagents and chemicals

Pharmaceutical grade pure Losartan Potassium, Ramipril and Hydrochlorothiazide gift samples were procured from Mylan Laboratories, Hyderabad. Marketed formulation Tablets with dose of 50mg of Losartan, 5mg of Ramipril and 12.5mg of Hydrochlorothiazide were procured from local market. (Mfd.by Unichem Laboratories). HPLC grade acetonitrile and Water were procured from Merck specialties private limited, Mumbai.

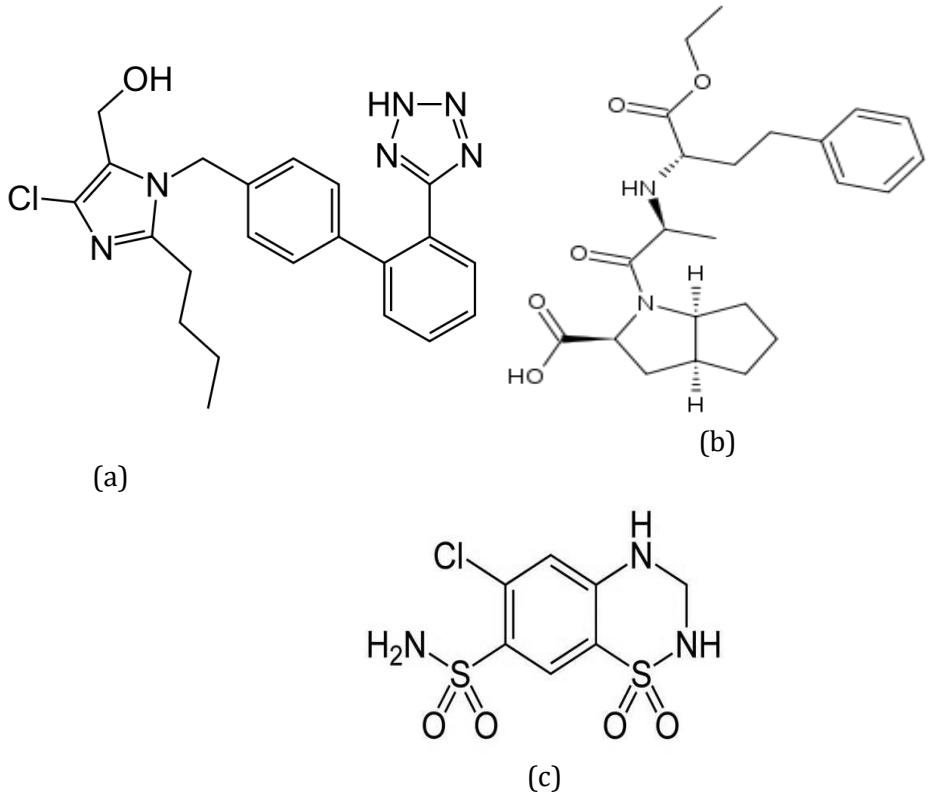

Figure 1: chemical structures of a) Losartan b) Ramipril c) Hydrochlorothiazide

Chromatographic conditions

Kromasil 100-5C_{18} column [250mm x 4.6mm] was used for the chromatographic separation at a detection wave length of 233 nm. Mobile phase of composition methanol: phosphate buffer-pH 4.0 in a ratio of 70:30 v/v was selected for elution and same mixture was used in the preparation of standard and sample solutions. Flow rate was adjusted to 0.8ml/min and the injection volume was 20µl.

Preparation of phosphate buffer (pH-4)

The buffer solution was prepared by dissolving accurately weighed amount of 7.0 grams of potassium dihydrogen phosphate into 1000ml HPLC grade water. Final pH was adjusted with ortho phosphoric acid.

Preparation of mobile phase

Mobile phase was prepared by mixing acetonitrile and buffer in the ratio of 70:30 v/v and was initially filtered through 0.45µm Millipore membrane filter and sonicated for 15 min before use for the elimination of any air bubbles if present.

Preparation of standard solutions

25mg each of Losartan Potassium, Ramipril and Hydrochlorothiazide were accurately weighed and transferred into three 25ml volumetric flasks, dissolved using mobile phase and the volume was made up with the same solvent to obtain primary stock solutions A (Losartan Potassium) ,B (Ramipril) and C

(Hydrochlorothiazide) of concentration 1000μg/ml of each drug. From the primary stock solutions , 1ml of each were pipette out from A, B and C respectively, transferred to a 10ml volumetric flask and the volume was made up with the mobile phase to obtain final concentrations of 100 μg/ml of each drug individually and this solution is (working stock solution A).

Preparation of sample solution

Twenty tablets of Losartan Potassium, Ramipril and Hydrochlorothiazide were weighed and crushed. Tablet powder equivalent to 50mg of Losartan, 5mg of Ramipril and 2.5mg of Hydrochlorothiazide was weighed accurately and transferred to a 50ml volumetric flask. The content was dissolved with 10ml of mobile phase and then sonicated for 15min. The volume was made up with the mobile phase and filtered with 0.45μmembrane filter and sonicated for 20min. 1ml of this solution was pipetted out and transferred to a 10ml volumetric flask and the volume was made up with the mobile phase to obtain a concentration of 1mg/ml of Losartan Potassium, 100μg/ml of Ramipril and 250μg/ml of Hydrochlorothiazide (working stock solution B).

Optimization of RP-HPLC method

The HPLC method was optimized with an aim to develop a simultaneous estimation procedure for the assay of Losartan Potassium, Ramipril and Hydrochlorothiazide. For the method optimization, different mobile phases were tried, but acceptable retention times, theoretical plates and good resolution were observed with methanol, phosphate buffer pH4 (70:30 v/v) using Kromasil 100-5C_{18} column [250mm x 4.6mm].

Validation of the RP-HPLC method

Validation of the optimized method was performed according to the ICH Q2 (B) guidelines.

System suitability

System suitability was carried out with five injections of solution of 100% concentration having 500 μg/ml of Losartan Potassium, 50μg/ml of Ramipril and 100 μg/ml of Hydrochlorothiazide in to the chromatographic system.

Linearity

For the determination of linearity, appropriate aliquots were pipetted out from working stock solution A to a series of 10ml volumetric flasks and volume was made up with the solvent to obtain concentration ranging from 100-500μg/ml of Losartan Potassium, 10-50μg/ml of Ramipril and 20-100μg/ml of Hydrochlorothiazide. Each solution was injected in triplicate. Calibration curves were plotted with observed peak areas against concentration followed by the determination of regression equations and calculation of the correlation coefficients. The calibration curves for Losartan Potassium, Ramipril and Hydrochlorothiazide were shown in Figure 3, 4 and 5. Their corresponding linearity parameters were given in Table 1.

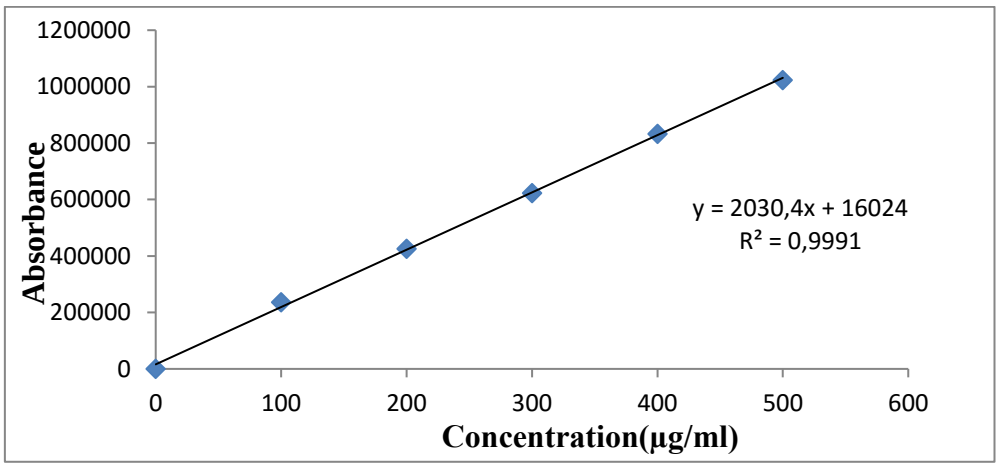

Figure 3: Calibration plot of Losartan

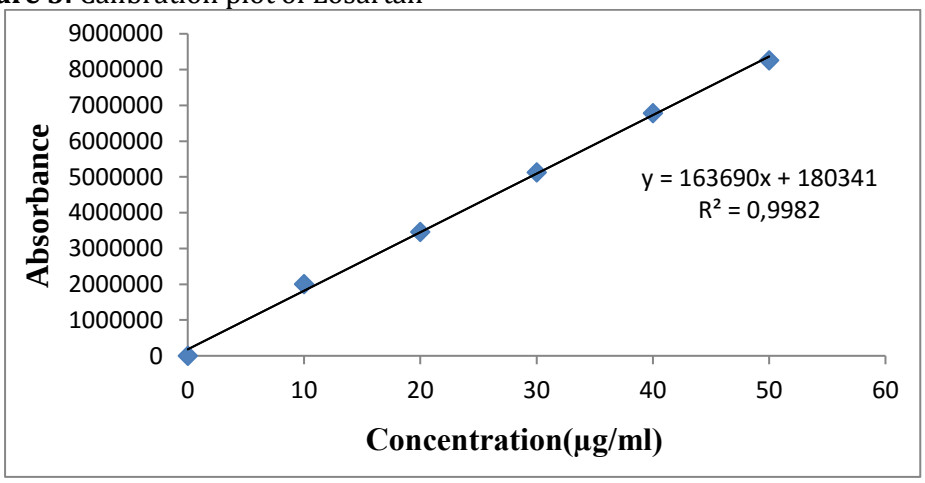

Figure 4: Calibration plot of Ramipril

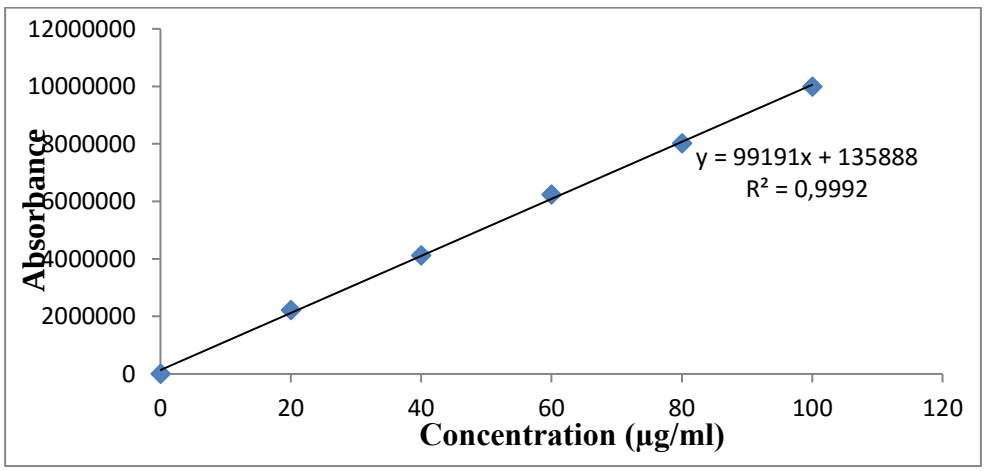

Figure 5: Calibration plot of Hydrochlorothiazide

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ were calculated from the slope(s) of the calibration plot and the standard deviation (SD) of the peak areas using the formula LOD = 3.3 σ/s and LOQ = 10 σ/s. The results were given in Table 1.

Precision

The repeatability of the method was verified by calculating the %RSD of six replicate injections of 100% concentration (500μg/ml of Losartan Potassium, 50μg/ml of Ramipril and 100μg/ml of Hydrochlorothiazide) on the same day and for intermediate precision % RSD was calculated from repeated studies on different days. The results were given in Table 2.

Accuracy

To ensure the reliability and accuracy of the method recovery studies were carried out by standard addition method. A known quantity of pure drug was added to pre-analyzed sample and contents were reanalyzed by the proposed method and the percent recovery was reported. The results were given in Table 2.

Specificity

Specificity of a method was determined by testing standard substances against potential interferences. The method was found to be specific when the test solution was injected and no interferences were found because of the presence of excipients. The optimized chromatogram of Losartan Potassium, Ramipril and Hydrochlorothiazide without any interference was shown in Figure 2.

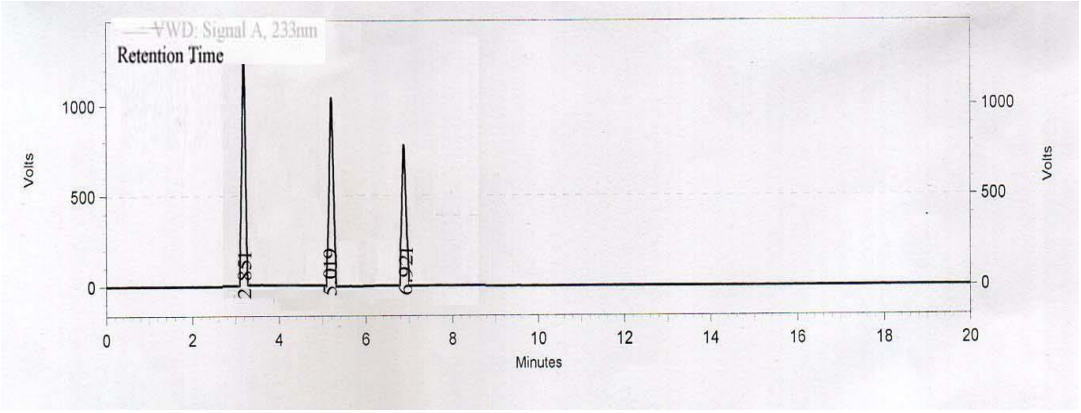

Figure 2: Optimized chromatogram of Losartan, Ramipril and Hydrochlorothiazide

Robustness

Robustness of the method was verified by altering the chromatographic conditions like mobile phase composition, wave length detection, flow rate, etc. and the % RSD should be reported. Small changes in the operational conditions were allowed and the extent to which the method was robust was determined. A deviation of ±2nm in the detection wave length and ±0.2ml/min in the flow rate, were tried individually. A solution of 100% test concentration with the specified changes in the operational conditions was injected to the instrument in triplicate. %RSD was reported in the Table 3.

Assay of marketed formulations

20µl of sample solution of concentration 10µg/ml of Losartan Potassium, 1µg/ml of Ramipril, and 2.5µg/ml of Hydrochlorothiazide was injected into chromatographic system and the peak responses were measured. The solution was injected three times in to the column. The amount of drug present and percentage purity was calculated by comparing the peak areas of the standards with that of test samples. A typical chromatogram for assay of marketed formulation was shown in Figure 6 and the obtained values were reported in the Table 5.

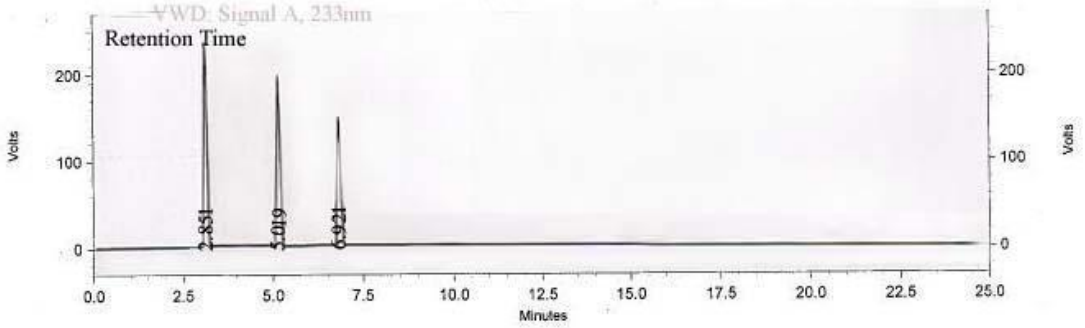

Figure 6: A typical chromatogram for assay of marketed formulation containing 10µg/ml of Losartan,1µg/ml of Ramipril and 2.5µg/ml Hydrochlorothiazide

FORCED DEGRADATION STUDIES

Acid degradation studies

Prepare each 1mg/ml stock solution of Losartan Potassium, Ramipril and Hydrochlorothiazide by using mobile phase as solvent then filtered through 0.45µm membrane filter paper. Stock solutions of Losartan Potassium 5ml, 0.5ml Ramipril and 1ml of Hydrochlorothiazide stock solution was transferred into 10ml volumetric flask and added 1 ml of 0.1 N HCL and diluted to volume with mobile phase. The resultant solution was injected into the system.

Alkaline degradation studies

Losartan Potassium, Ramipril and Hydrochlorothiazide primary stock solutions were prepared with mobile phase and then filtered through 0.45µm membrane filter paper. Stock solutions of 5 ml and 1ml of Losartan Potassium, Ramipril and Hydrochlorothiazide stock solutions of highest concentration in the linearity range prepared into 10ml volumetric flask and added 1 ml of 0.1 NaOH and diluted to volume with mobile phase.

Oxide degradation studies

To know the oxidative degradation in the selected drugs of choice 1mg/ml of stock solution of three drugs were prepared then filtered through 0.45µm membrane filter paper. Stock solutions of 100µg/ml of Losartan Potassium, 50µg/ml of Ramipril and 100µg/ml of Hydrochlorothiazide were prepared into 10ml volumetric flask and added 1 ml of H_2O_2 and diluted to volume with mobile phase.

Thermal degradation studies

Prepared each 1mg/ml of stock solution with Losartan Potassium, Ramipril and Hydrochlorothiazide stock solution by diluting with mobile phase as solvent and

was then filtered through 0.45µm membrane filter paper. Stock solutions of 5 ml and 1ml of Losartan, Ramipril and Hydrochlorothiazide in 10ml volumetric flask was prepared by transferring and kept for 60^0c at hot air oven. From the obtained chromatogram it was proved that the selected samples were stable against thermal conditions. The chromatogram was shown in Figure 6.

RESULTS AND DISCUSSION

After a number of trials with mobile phases of different composition, Methanol, Phosphate buffer pH 4 in the ratio 70:30v/v was selected as mobile phase because of better resolution and symmetric peaks. Losartan Potassium, Ramipril and Hydrochlorothiazide were found to show appreciable absorbance at 233nm when determined spectrophotometrically and hence it was selected as the detection wavelength. An optimized chromatogram showing the separation of Losartan Potassium, Ramipril and Hydrochlorothiazide at different R_Ts of 2.851, 5.019 and 6.921 were shown in Figure 2. System suitability was carried out by injecting 5 replicate injections of 100% test concentration, number of theoretical plates HETP (Height Equivalent to the Theoretical Plate) and resolution were satisfactory. The chromatograms confirm the presence of Losartan Potassium, Ramipril and Hydrochlorothiazide at 2.85, 5.019min and 6.92min respectively without any interference. Concentration range of 100-500µg/ml for Losartan Potassium, 10-50µg/ml for Ramipril and 20-100µg/ml for Hydrochlorothiazide were found to be linear with correlation coefficients 0.999, 0.998 and 0.999 for Losartan Potassium, Ramipril and Hydrochlorothiazide respectively. The results were given in Table 1. The limits of detection for Losartan Potassium, Ramipril and Hydrochlorothiazide were found to be 3.0µg/ml,4.8µg/ml and 5.2µg/ml respectively and the limits of quantitation were 10.7µg/ml, 9.32µg/ml and 15.7µg/ml respectively. Values were represented in Table 1.

The proposed method was found to be precise and reproducible with %RSD of 0.79, 0.82 and 0.73 for Losartan, Ramipril and Hydrochlorothiazide respectively. %RSD was reported in Table 2.

Table 1: Results for Linearity (n=3)

Parameters	Losartan	Ramipril	Hydrochlorothiazide
Slope	20304	163690	99191
y intercept	20304+16024=36328	163690+180341=344031	99191+13588=112779
Correlation coefficient r^2	0.999	0.998	0.999
Regression Equation	Y=20304x+16024	Y=163690x+180341	Y=99191x+13588
Linearity range	100-500µg/ml	10-50µg/ml	20-100µg/ml
LOD	3.0µg/ml	4.8µg/ml	5.2µg/ml
LOQ	10.7µg/ml	9.32µg/ml	15.7µg/ml

∗n= No. of determinants

Table 2: Results of precision (n=6)

Drug	Intraday Precision (%RSD)	Interday Precision (%RSD)
Losartan	0.79	0.82
Ramipril	0.82	0.96
Hydrochlorothiazide	0.73	0.89

∗n= No. of determinants

Accuracy of the method was verified by performing recovery studies by standard addition method. The percent recovery of the standard added to the pre-analysed sample was calculated and it was found to be 99.2% to 100.07% for Losartan

Potassium, 99.6-99.9% for Ramipril and 99.5-100.15 % for Hydrochlorothiazide. This indicates that the method was accurate. Values obtained were given in Table 3. The method was found to be robust after changing the conditions like detection wavelength (± 2nm) and flow rate (± 0.2 ml). %RSD was calculated for each variation and reported. Values obtained were given in Table 4. The method was found to be specific for the combination of interest after verifying the chromatograms showing no interference of the excipients present. Hence, the method was well suitable for the estimation of the commercial formulations of the selected combination with a percentage purity of 100.76% for Losartan Potassium, 96% for Ramipril and 98.82% for Hydrochlorothiazide. The typical chromatogram for assay of marketed formulations was shown in Figure.5 and Values obtained were given in Table 5.

Degradation studies indicated the specificity of developed method in presence of degradation products. Degradation was carried out in combination of three drugs and purity of drug peaks was confirmed by purity angles. According to current good manufacturing practice (cGMP), all drugs must be tested under a stability-indicating method before release. Stress testing of drug substance can help in identifying the likely degradation products which can establish the degradation pathways and the intrinsic stability of the molecule. The nature of the stress testing will depend on the individual drug substance. These studies provide valuable information on drugs inherent stability and help in the validation of analytical methods to be used in stability studies. The chromatograms obtained from samples treated with acid, base, hydrogen peroxide, heat and photo degradation were examined. The chromatograms showed that Losartan Potassium, Ramipril and Hydrochlorothiazide were degraded under acidic, basic and peroxide conditions. Degradation was not observed for Losartan Potassium, Ramipril and Hydrochlorothiazide samples during stress conditions like heat and photo degradation, they show only one peak of drug under these stress conditions. The results of the stress studies indicated the specificity of the developed method. Losartan Potassium degraded more in base than in acid and oxidative stress conditions, whereas Ramipril degraded more in oxidative than other conditions. Hydrochlorothiazide was found to be more stable than Losartan Potassium and Ramipril in all three conditions. The typical chromatogram of thermal degradation was shown in Figure 7.

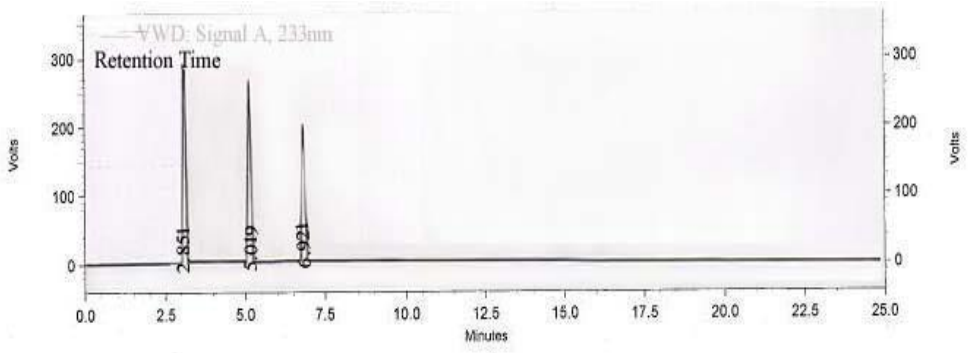

Figure 7: Chromatogram of thermal degradation

Table 3: Results for Accuracy (n=3)

Recovery level	Amount of Standard drug added (µg/ml)			Amount of test added (µg/ml)			Total Amount Recovered (µg/ml)			% Recovery w/w		
	LOP	RAM	HTZ	LOP	RAM	HTZ	LOP	RAM	HTZ	LOP	RAM	HTZ
80%	100	10	20	300	30	60	299.2	39.6	79.5	99.2	99.6	99.6
100%	200	20	40	300	30	60	499.4	49.8	99.5	99.5	99.8	99.5
120%	300	30	60	300	30	60	599.9	60.5	120.2	100.07	99.9	100.15

∗n= No. of determinant

Table 4: Results for Robustness (n=3)

Parameters (n=3)	%RSD		
	Losartan	Ramipril	Hydrochlorothiazide
Detection wavelength at 231nm	0.94	0.32	0.33
Detection wavelength at 235nm	0.45	0.87	0.70
Flow rate 0.6ml/min	0.35	0.21	0.12
Flow rate 1.0ml/min	0.18	0.58	0.18

∗n= No. of determinant

Table 5: Results for Assay (n=3) of Marketed formulation

Drug	Label claim (mg/tab)	Amount recovered	% Amount found in drug
Losartan	50	50.38	100.76%
Ramipril	5	4.8	96%
Hydrochlorothiazide	12.5	12.22	98.22%

∗n= No. of determinants

CONCLUSION

The RP-HPLC method developed and validated allows a simple and fast quantitative determination of Losartan Potassium, Ramipril and Hydrochlorothiazide from their formulations. All the validation parameters were found to be within the limits according to ICH guidelines. The proposed method was found to be specific for the drugs of interest irrespective of the excipients present and the method was found to be simple, accurate, precise, rugged, robust, economical ecofriendly and stable under forced degradation stress conditions. So the established method can be employed in the routine analysis of the marketed formulations.

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