

# Stability-indicating Reversed-phase Liquid Chromatographic Method for Simultaneous Determination of Losartan Potassium and Ramipril in Tablets

S. KOLLIPARA, G. BENDE, Y. BANSAL<sup>1</sup> AND R. SAHA\*

Formulation Development and Pharmacokinetics Laboratory, Pharmacy Group, Birla Institute of Technology and Science, Pilani-333 031, <sup>1</sup>IPCA Laboratories Limited, Kandivli Industrial Estate Ltd., Kandivli (West), Mumbai-400 067, India

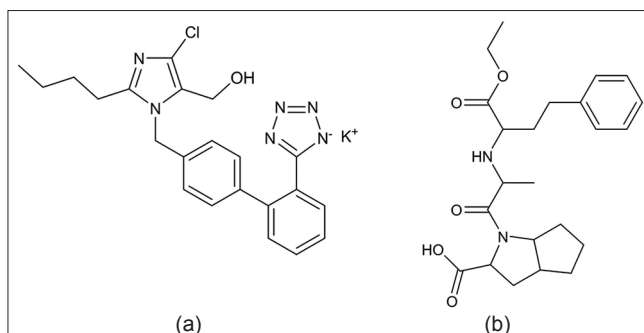
Kollipara, *et al.*: RP-LC Method for Determination of Losartan and Ramipril

A stability-indicating reversed-phase liquid chromatographic method has been developed and validated for simultaneous determination of losartan potassium and ramipril. Separations were achieved using a C<sub>18</sub> column with mobile phase consisting of acetonitrile and (0.2% v/v, pH 2.5) aqueous trifluoroacetic acid (45:55, v/v) in isocratic mode at 1 ml/min flow rate. Column effluent was monitored at 210 nm using a UV detector. The method was validated for selectivity, linearity, accuracy, precision, sensitivity and robustness. Novel microwave-assisted forced degradation technique was employed for evaluation of selectivity. The method demonstrated excellent linearity for losartan potassium and ramipril with regression coefficients of 0.9999 and 0.9998, respectively. The linearity range was found to be 62.5-5000 ng/ml and 125-10,000 ng/ml with the mean percentage recoveries of 100.36% ( $\pm 2.27$ ) and 100.16% ( $\pm 3.33$ ) for losartan potassium and ramipril, respectively. In a robustness study, a full factorial design revealed that the analytical response remains unaffected by small variations in the critical chromatographic factors. The method was found to be sensitive with quantification limits of 44.30 and 79.93 ng/ml for losartan potassium and ramipril. The method was successfully employed for the determination of losartan potassium and ramipril in commercially available and in-house prepared tablets.

**Key words:** Design of experimentation, losartan potassium, microwave-assisted degradation, ramipril, robustness testing

Losartan potassium (LS), chemically 2-butyl-4-chloro-1-[p-(o-1H-tetrazol-5-ylphenyl) benzyl] imidazole-5-methanol mono potassium salt (fig. 1a), is a non-peptide angiotensin II receptor (type AT1) antagonist. LS is widely used in the treatment of high blood pressure and diabetic nephropathy in patients with type 2 diabetes mellitus (T2DM). Ramipril (RM), chemically (2S,3aS,6aS)-1[(S)-N-[(S)-1-carboxy-3-phenylpropyl] alanyl] octahydrocyclopenta[b] pyrrole-2-carboxylic acid, 1-ethyl ester (fig. 1b), is a non-sulfhydryl angiotensin converting enzyme inhibitor. RM is the drug of choice for patients at a high risk of developing major cardiovascular complications arising from a history of coronary artery disease, stroke, peripheral vascular disease and T2DM

associated with cardiovascular risk. The inhibition of the renin-angiotensin-aldosterone system, using an angiotensin-converting enzyme inhibitor such as RM, with a selective angiotensin receptor AT1 blocker such as LS, is proposed as a novel therapeutic strategy to reduce cardiovascular mortality. Co-administration of LS and RM exerts favourable metabolic effects, which aid in prevention of T2DM. Recently, a fixed-dose



**Fig. 1:** Chemical structures of analytes. Chemical structures of (a) losartan potassium and (b) ramipril

\*Address for correspondence

E-mail: rnsaha@bits-pilani.ac.in

combination therapy of LS and RM was approved in the Indian and Asian markets for the management of hypertension in patients with associated T2DM<sup>[1]</sup>.

Extensive literature survey did not reveal a simple, selective and sensitive analytical method for simultaneous determination of LS and RM. Most of the methods are reported for the determination of either LS or RM, separately. A few methods are also reported for simultaneous determination of either LS or RM with hydrochlorothiazide. However, several reported methods are developed for biological applications such as serum, plasma, urine, bile and other tissues<sup>[2-11]</sup>. Moreover, most of the methods either demonstrate limitations such as poor sensitivity, selectivity, repeatability or use sophisticated analytical techniques such as LC-MS/MS, HPTLC, CE and SCF, thus making them unsuitable for routine analysis<sup>[12-20]</sup>. Literature survey revealed a single method for simultaneous determination of LS and RM with hydrochlorothiazide, however, this method is not stability-indicating and lacks in chromatographic resolution between LS and solvent front<sup>[21]</sup>.

Chromatographic separation and simultaneous quantification of LS and RM is challenging, as the molar extinction coefficient and aqueous solubility of RM is poor in comparison with LS. In addition, the low-dose proportion of RM to LS does not allow unified dilution scheme as LS concentration remains above calibration levels with low dilution and RM concentration falls below calibration levels with high dilution. All these challenges were considered in the present study and a stability-indicating method has been developed and validated for simultaneous determination of LS and RM in the presence of their degradation products. Novel microwave-assisted degradation technique was used for forced degradation studies<sup>[22]</sup>. Mobile phase was optimised for simple isocratic elution system with cost-effective combination. Additionally, the design of experimentation (DoE) technique was employed to study the effect of critical factors on the method performance.

## MATERIALS AND METHODS

Losartan potassium (assay 99.95%), Ramipril (assay 99.95%) were kindly gifted by IPCA Laboratories Ltd., India. Disodium hydrogen phosphate, potassium dihydrogen phosphate and ammonium phosphate

analytical-reagents were purchased from S.D. Fine Chemicals Ltd., India. HPLC-grade solvents, acetonitrile, methanol, triethylamine and trifluoroacetic acid were purchased from Spectrochem, India. Various excipients present in formulation like microcrystalline cellulose, lactose (hydrous), pre-gelatinised starch, magnesium stearate and iron oxide were obtained from Medreich Pharmaceuticals, India. Ultrapure water was prepared using a MilliQ<sup>®</sup> water purification system (Millipore Co, Billerica, USA) and filtered through a 0.22- $\mu\text{m}$ -pore-size filter before use. All other chemicals used in the analysis were of analytical-reagent grade. One commercially available tablet of LS and RM was selected from local Indian market and one in-house tablet formulation was prepared containing common excipients.

### Chromatographic system and conditions:

An HPLC system (Jasco, Kyoto, Japan) consisted of PU-1580 series binary system, AS-1559 autosampler and UV-1575 series UV/Vis detector was used for chromatographic determinations. Optimised mobile phase consisting of acetonitrile and (0.2% v/v, pH 2.5) aqueous trifluoroacetic acid (45:55, v/v) was degassed under vacuum and delivered in isocratic elution mode at a flow rate of 1 ml/min. The chromatographic separations were carried out using a Hibar<sup>®</sup> (Merck, Mumbai, India) C<sub>18</sub> reverse phase endcapped column (250×4.6 mm, 5  $\mu\text{m}$ , 100 Å). The quantification was carried out using UV-detector at 210 nm with injection volume of 50  $\mu\text{l}$ . Chromatographic peak integration was performed using BORWIN<sup>®</sup> work station (Jasco). All experiments were carried out at ambient temperature after baseline stabilisation.

### Stock solutions and standards:

Stock solution of 1 mg/ml was prepared by dissolving 100 mg of LS or RM in 100 ml of dilution solution consisting of acetonitrile and MilliQ water (50:50, v/v). Prepared standards were sonicated for 15 min. Secondary stock solution of 10 and 5  $\mu\text{g}/\text{ml}$  were prepared by diluting 1.0 and 0.5 ml of standard stock solutions to 100 ml in mobile phase for LS and RM, respectively.

A series of seven calibration standard solutions containing 62.5, 125, 250, 500, 1000, 2500 and 5000 ng/ml of LS and 125, 250, 500, 1000, 2500, 5000 and 10,000 ng/ml of RM were prepared by transferring 0.125, 0.25, 0.5, 1.0, 2.5, 5 and 10 ml

of the secondary stock solution into 10-ml calibrated flasks and diluting to volume with mobile phase. Mixed standard solutions of LS and RM (62.5 and 125 ng/ml, 500 and 1000 ng/ml, 5000 and 10,000 ng/ml,) were also prepared from secondary stock solutions by suitable dilution in mobile phase.

#### **Spiked placebo standards:**

Six different series of sample standards were prepared by adding known amount of drug in placebo blend at three levels 80, 100 and 120% of the labelled claim of the tablet (50 mg of LS and 5 mg of RM) and analysed by the proposed method. For each series, blend of excipients present in formulation consisting of colloidal silicon dioxide (NF), crospovidone (NF), hypromellose (USP), magnesium stearate (NF) and microcrystalline cellulose (NF) was prepared by replacing drugs with lactose. Placebo blend equivalent to one tablet was weighed and transferred into a 100 ml volumetric flask and the amount of LS and RM at 80, 100 and 120% labelled claim of tablet were added to it and the volume was made up to 100 ml with dilution solution. Prepared sample standards were filtered through Whatman® filter paper after sonication for 15 min. Finally, 50 µl of prepared solution was transferred to a 10 ml calibrated flask and diluted to volume with mobile phase.

#### **Method validation:**

The developed analytical method was validated as per ICH and USP guidelines and the studied parameters are presented below<sup>[23,24]</sup>.

#### **Selectivity:**

For the assessment of selectivity, six different placebo tablets were processed and analysed by the proposed method. Additionally, sample standards (placebo spiked) at LOQ level of LS and RM were analysed in six replicates and compared with fresh calibration standards to evaluate interference in determination of LS and RM. Further, forced degradation studies were carried out to assess the selectivity of the method.

Forced degradation studies of LS and RM were performed by exposing individual drugs to hydrolytic, photolytic, thermal and oxidative stress conditions. A novel microwave-assisted forced degradation technique was employed for hydrolytic stress testing<sup>[22]</sup>. For each stress treatment, 10 mg of the drug was accurately weighed and transferred to a 10 ml volumetric flask. For hydrolytic treatment,

2 ml of either MilliQ water or 2 M hydrochloric acid or 2 M sodium hydroxide was added to each flask containing drug. All samples were vortex-mixed and subjected to microwave radiation (15 s/cycle, 2.45 GHz, 300 W, 80% intensity) for 5 min. Samples were allowed to cool and neutralised.

For photolytic treatment, 2 ml of MilliQ water was added to each volumetric flask containing drug and samples were subjected to natural sunlight for 12 h (6 h per day). For thermal stress treatment, 2 ml of MilliQ water was added to volumetric flask containing the drug and subjected to 90° for 12 h. For oxidative treatment, 2 ml of hydrogen peroxide (3%, v/v) solution was added to volumetric flask containing drug. Samples were vortex-mixed and kept on a mechanical shaker at room temperature for 12 h at 25°, protected from light. After each treatment, samples were suitably diluted and analysed by the proposed method. For better understanding of the selectivity of method, an orthogonal method was developed by changing the relative proportion of organic phase to (30%, v/v) while keeping all other chromatographic conditions constant<sup>[25]</sup>. All forced degradation samples were also subjected to orthogonal separation for selectivity estimation.

#### **Linearity and range:**

For assessment of the linearity of response, three individual series of calibration standards at seven levels of LS (62.5-5000 ng/ml) and RM (125-10,000 ng/ml) were injected in six replicates. The calibration curves were obtained by plotting mean peak area against the concentration using linear regression analysis. Analysis of residuals and one-way ANOVA was performed for replicate measurements of peak area obtained at each concentration<sup>[26]</sup>.

#### **Accuracy:**

Accuracy of the method was evaluated by performing recovery experiments in six replicates for three different days. The recovery experiments were conducted at 80, 100 and 120% of labelled claim of tablet by adding known amount of drugs to the placebo blend. At each level, six samples were analysed on three different days and the percentage recoveries were calculated using the fresh calibration curve.

#### **Repeatability:**

Repeatability of the method was determined by performing six replicate injections of freshly prepared

sample standards of LS and RM at three levels 80, 100, and 120% of the labelled claim of the tablet. The relative standard deviation (RSD) of response was determined at each concentration level.

#### Intermediate precision:

The intermediate precision of the method was determined by performing six independent replicate injections of LS and RM at three levels 80, 100 and 120% of the labelled claim of the tablet on three different days. The RSD values at each concentration level were determined.

#### Sensitivity:

The sensitivity of the method was determined by calculating the limit of detection (LOD) and limit of quantification (LOQ). The standard deviation of intercept ( $\sigma$ ) and slope of calibration curve ( $s$ ) were used for the calculation of LOD ( $3.3 \sigma s^{-1}$ ) and LOQ ( $10 \sigma s^{-1}$ ) for both the drugs.

#### Robustness testing:

The DoE technique was employed to identify critical chromatographic factors and to study their effect on the method parameters. Critical sources of variability from the operating procedure were identified and investigated in the range that covers the variation due to intra and inter-laboratory conditions. Three selected factors were pH of aqueous phase (pH:  $X_1$ , 2.0 and 3.0, adjusted with acetic acid), percentage of trifluoroacetic acid in aqueous component (% TFA:  $X_2$ , 0.1 and 0.3%, v/v) and aqueous phase percentage in mobile phase (% AQ:  $X_3$ , 52.5 and 57.5%, v/v). Experimental plan ( $2^3$  full-factorial design) and the domain of selected variables is shown in Table 1. Experimental design comprising of eight experiments were conducted in random order and all other parameters were kept constant. For each experiment, triplicates injections of LS (5000 ng/ml)

and RM (10,000 ng/ml) were made after baseline stabilisation. Each experiment was conducted in triplicate and chromatographic parameters (peak area and retention time) with system suitability test (asymmetry factor and resolution) were recorded as experimental response. The model coefficients were obtained by the least square regression analysis. These model coefficients were used for response surface analysis (Design Expert®-demo version 7.1.4, Stat Ease) by plotting predicted responses using following equation:

$$\hat{Y} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{23} X_2 X_3 + \beta_{31} X_3 X_1 + \beta_{123} X_1 X_2 X_3 \quad (1)$$

where  $\hat{Y}$ =predicted response,  $X$ =coded variable, and  $\beta$ =model coefficient.

#### System suitability:

For system suitability study, six replicate injections of mixed standard solutions were injected and capacity factor ( $K'$ ), asymmetry factor ( $A_s$ ), number of theoretical plates ( $N$ ), height equivalent to theoretical plates (HETP) and resolution ( $R_s$ ) were calculated for both the drugs.

#### Sample solution stability:

The stability of the both drugs in mobile phase was tested by injecting sample standard at 100% level of labelled claim in triplicates at intervals of 6, 12, 24, 48 and 72 h. The mean peak areas at each time point were compared against freshly prepared standards.

#### Determination of LS and RM in tablets:

The proposed method was employed for the determination of LS and RM drug content in real world samples such as marketed tablet formulation (Loram-5; Kalindi Medicare Pvt. Ltd., Mumbai, India containing 50 mg of LS and 5 mg of RM) and in-house prepared tablet formulation (containing

**TABLE 1: EXPERIMENTAL PLAN USING  $2^3$ -FULL FACTORIAL DESIGN FOR THE ROBUSTNESS TESTING**

Exp. no.	Sequence no.	Critical factors			Response for LS			Response for RM			$R_s$
		pH ( $X_1$ )	% TFA ( $X_2$ )	% AQ ( $X_3$ )	PA	$R_t$	$A_s$	PA	$R_t$	$A_s$	
1	6	+1	+1	+1	867,354	8.08	1.10	631,915	10.32	1.25	5.58
2	3	+1	+1	-1	918,166	6.27	1.12	672,942	7.71	1.26	4.97
3	1	+1	-1	+1	976,166	9.02	1.12	555,635	13.67	1.49	9.19
4	5	+1	-1	-1	981,939	6.63	1.17	558,998	9.94	1.42	8.90
5	7	-1	+1	+1	928,497	6.66	1.16	594,233	10.20	1.26	9.78
6	4	-1	+1	-1	953,470	5.51	1.19	646,530	7.88	1.29	7.81
7	8	-1	-1	+1	1,011,999	7.23	1.92	579,030	11.81	1.83	8.77
8	2	-1	-1	-1	904,377	5.85	1.40	656,926	8.95	1.61	6.66

$2^3$ -full factorial design with three selected critical factors at two levels ( $X_1$ -2.0, 3.0;  $X_2$ -0.1, 0.3 v/v and  $X_3$ - 52.5, 57.5 v/v) for the robustness testing and mean recorded responses for losartan potassium (LS) and ramipril (RM). PA=Peak area,  $R_t$ =Retention time,  $A_s$ =Asymmetry factor,  $R_s$ =Resolution

50 mg of LS and 5 mg of RM). For the estimation of drug content in tablet, the average weight of twenty tablets was noted, tablets were powdered and mixed uniformly. A quantity of powder equivalent to one tablet was accurately weighed and processed as per procedure described in sample standards section. Finally, 50  $\mu$ l of resulting solution was injected in triplicates and analysed by proposed method. The mean drug content for both the drug was determined using calibration curve.

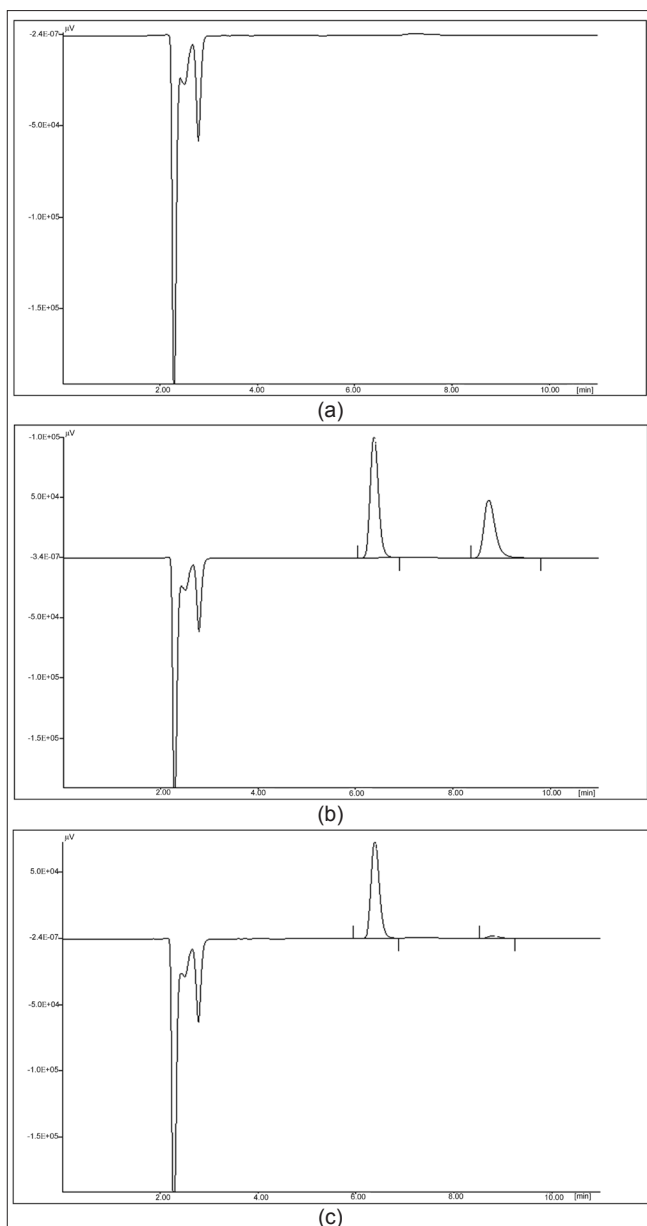
## RESULTS AND DISCUSSION

During initial trials, chromatographic conditions such as composition of mobile phase, buffering salt, buffer pH and ionic strength were optimised for better retention and peak properties of both the drugs. Although, the UV absorption spectrum of LS and RM have shown wavelength maxima at 210 nm, RM demonstrated extremely poor molar absorptivity. Moreover, commercial formulations containing a high proportion of LS and low proportion of RM at a fixed dose combination (LS: 50 mg and RM: 5 mg) offered a narrow dilution window. Accurate and precise quantification RM was a critical step for successful method development as the narrow dilution window along with poor molar absorptivity of RM presented a significant challenge in simultaneous determination of RM and LS. In preliminary studies, the wavelength was optimised to 210 nm for maximum sensitivity for RM determination, as detection at other wavelengths showed poor quantification limits. Mobile phase consisting of acetonitrile and MilliQ water (50:50, v/v) demonstrated lower retention time ( $R_t$ ) for LS ( $R_t=1.7$  min); whereas RM demonstrated extensive retention ( $R_t>30$  min) with poor peak properties. The effect of mobile phase pH on retention time and peak properties were studied with ammonium acetate buffer. Acetonitrile and (20 mM, pH 3.5) ammonium acetate buffer (50:50, v/v) showed good retention of LS ( $R_t=5.5$  min) with moderate peak properties. However, RM showed extensive retention with all combinations of pH (3.5, 4.5 and 5.5) and organic to aqueous phase ratio (30:70, 50:50 and 70:30, v/v). Ammonium phosphate buffer at 20 mM strength and pH 3.5, 4.5, 5.5 and 6.5 with (0.01%, v/v) triethylamine improved the retention and peak properties for LS ( $R_t=6.6$  min) but resulted in extensive retention of RM with moderate peak properties, which was inadequate even at higher proportions of organic modifier. Acetonitrile and (1%,

v/v) aqueous acetic acid (50:50, v/v) demonstrated better resolution with improved peak properties for both the drugs. However, the noise was significantly high which may be attributed to increased absorbance of mobile phase at 210 nm. Addition of trifluoroacetic acid (TFA) in aqueous phase (0.1%, v/v) with acetonitrile (50:50, v/v) showed improved peak properties for RM with better detection limits and retention for LS and RM. Finally, mobile phase was optimised to acetonitrile and (0.2%, pH 2.5) aqueous trifluoroacetic acid (45:55, v/v) for better resolution and peak properties with significantly low noise.

Placebo tablets analysed by proposed method showed no significant interference in the determination of LS and RM at the detection wavelength of 210 nm. Moreover, sample standards (placebo spiked) prepared at LOQ level of respective drugs demonstrated no interference in determination of LS and RM when compared with calibration standards. Chromatograms for placebo blank, calibration standard, sample standard demonstrating selectivity of method for determination of LS and RM are shown in fig. 2.

Additionally, forced degradation samples analysed by proposed method proved that the degradation products of LS do not interfere in determination of RM and *vice versa*. No degradation products of either drug showed retention time in near vicinity of the other drug indicating the selectivity of the method for determination of LS and RM in presence of degradation products. LS showed highest sensitivity towards oxidative and neutral hydrolytic stress conditions leading to maximum degradation. Degradation in base hydrolytic condition showed one degradation product eluting at 13.5 min, whereas neutral and acid hydrolytic conditions showed lower degradation. LS showed lower degradation under thermal and photolytic stress conditions. Additionally, forced degradation samples subjected to orthogonal separation showed single sharp peak with retention time of 10.59 min suggesting absence of degradation products eluting at retention time of LS and selectivity of method. RM showed good stability in neutral hydrolytic and photolytic stress conditions whereas it showed complete degradation in base hydrolytic, acid hydrolytic and thermal stress conditions. Degradation in base hydrolytic and thermal stress conditions showed two degradation products eluting before 5 min, however, acid hydrolytic condition showed no degradation



**Fig. 2: Overlain chromatograms of samples. Chromatograms for (a) placebo blank, (b) calibration standard and (c) sample standard demonstrating selectivity of method**

products eluting for 30 min. Oxidative degradation samples showed moderate degradation of RM with three degradants eluting at 14.5, 15.5 and 17 min, respectively. Moreover, degradation samples subjected to orthogonal separation showed single sharp peak with average retention time of 22.2 min indicating the absence of degradants eluting at retention time of RM. Selected forced degradation samples demonstrating selectivity and stability-indicating ability of the method are given in fig. 3.

The calibration equation for LS and RM were found to be Peak Area = (259.86 × Conc. (ng/ml)) – 2059.5

and Peak Area = (82.37 × Conc. (ng/ml)) + 324.36 with regression coefficients of 0.9999 and 0.9998, respectively indicating the linear relationship between experimental response and concentration. The RSD of peak area at each concentration level was found to be significantly less (<3.02%). Analysis of residuals showed that the residuals were randomly distributed around zero with uniform variance across all concentration levels indicating homoscedasticity of the data. Finally, one-way ANOVA performed for peak area at each concentration level indicated that calculated *F*-value was less than critical *F*-value at 5% significance level.

The method has shown good and consistent recoveries at all three levels in placebo spiking method (Table 2). The mean absolute recovery of three levels for 18 determinations of LS and RM were 100.36 and 100.16%, respectively. The RSD values of absolute recoveries for LS and RM were found to be 2.27 and 3.33%, respectively. Low values of % bias demonstrated insignificant interference of excipients on the determination of either drug. Consistently high absolute recoveries demonstrated the suitability of the method for determination of LS and RM in tablet preparations.

Analysis of six replicate injections of freshly prepared LS and RM sample standards at three levels as a part of repeatability study proved that there is no significant variation in experimental response (Table 2). The RSD values were found to be significant at <1.44 (for LS) and 0.07% (for RM), respectively. Moreover, analysis of LS and RM sample standards on different days proved the inter-day precision of the method as RSD values were found to be significant at <0.92 (for LS) and 0.11% (for RM), respectively. Low values of RSD indicated the repeatability and intermediate precision of the proposed method.

The LOD and LOQ for LS were found to be 14.62 and 44.30 ng/ml, respectively. The LOD and LOQ for RM were found to be 26.37 and 79.93 ng/ml, respectively. Moreover, repeated injections performed at LOQ level of both the drugs showed less variation in experimental response with insignificant changes peak properties. The results indicate the sensitivity of the method in the determination of LS and RM in comparison with previously reported methods. The summary of validation parameters is listed in Table 3.

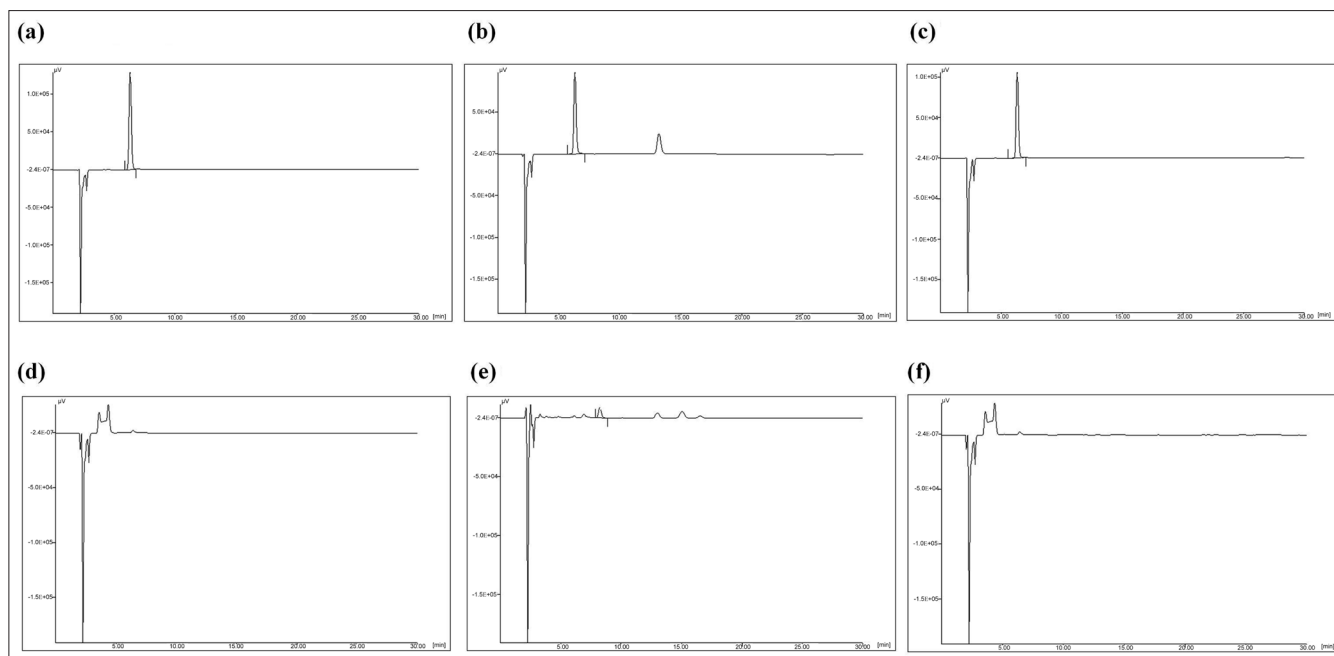


Fig. 3: Chromatograms of forced degradation studies.

Chromatograms indicate method's ability to demonstrate selectivity and stability indication for losartan potassium and ramipril subjected to various forced degradative conditions. Where, (a) acid hydrolytic condition for LS; (b) base hydrolytic condition for LS; (c) thermal degradation condition for LS; (d) acid hydrolytic condition for RM; (e) base hydrolytic condition for RM; (f) thermal degradation condition for RM. LS=losartan potassium and RM=ramipril.

TABLE 2: THE MEAN RECOVERIES AND RELATIVE STANDARD DEVIATION FOR LS AND RM

Drug	% Standard spiking in placebo (of label claim) <sup>a</sup>	Amount added (mg)	Amount recovered <sup>b</sup> (mg)	Recovery (%)	RSD (%)	Bias (%)
LS	80	40	40.37	100.93	0.21	0.92
	100	50	49.74	99.48	0.78	0.52
	120	60	60.40	100.67	1.44	0.67
RM	80	4	3.94	98.50	0.06	1.50
	100	5	4.99	99.80	0.07	0.20
	120	6	6.13	102.17	0.05	2.17

LS=Losartan potassium, RM=ramipril, RSD=Relative standard deviation. <sup>a</sup>Placebo tablet matrix equivalent to unit dose weight. <sup>b</sup>n=6 (six independent series were prepared and injected)

Analysis of three-dimensional surface plots generated from least square regression analysis proved that the experimental response remains unaffected by small but deliberate changes in the studied variables. The developed model showed high correlation between experimental response and critical variables. The obtained model coefficients were successfully used to evaluate the relationship between critical factors and response functions. The model coefficients used for response surface analysis are listed in Table 4.

Fig. 4 shows three-dimensional surface plots of predicted responses (on Y-axis) for LS and RM as a function of two significant factors (on X<sub>1</sub>- and X<sub>2</sub>-axis) whereas the third, the least significant factor

TABLE 3: SUMMARY OF VALIDATION PARAMETERS

Parameter	LS	RM
Calibration range	62.5-5000 ng/ml	125-10,000 ng/ml
Linearity (correlation coefficient)	0.9999	0.9998
Regression equation	Peak area= (259.86×Conc.)-2059.5	Peak area= (82.37×Conc.)+324.36
Confidence interval of slope ( $\alpha=0.05$ )	259.07-260.64	82.14-82.59
Confidence interval of intercept ( $\alpha=0.05$ )	-3755.04 to -364.04	-656.51 to 1305.23
Limit of detection (LOD)	14.62 ng/ml	26.37 ng/ml
Limit of quantification (LOQ)	44.30 ng/ml	79.93 ng/ml
Mean absolute recovery ( $\pm$ SD)	100.36% ( $\pm$ 2.27)	100.16% ( $\pm$ 3.33)
Precision (% RSD) repeatability	1.44	0.07
Precision (% RSD) intermediate precision	0.92	0.11

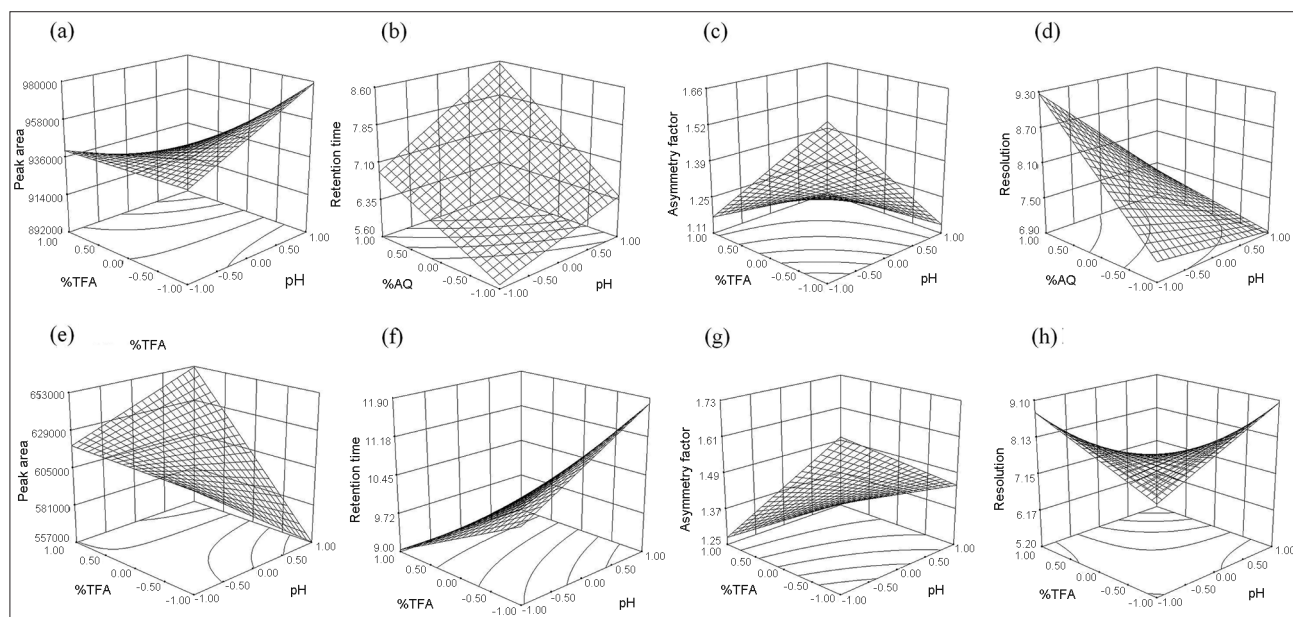
LS=Losartan potassium, RM=Ramipril, RSD=Relative standard deviation

is held constant at optimum level. The 3D surface plot (fig. 4a and e) shows peak area of LS and RM against pH and % TFA, where there is no significant change in the mean peak area over studied range of pH and % TFA indicating the robustness of chromatographic response for studied factors. % AQ

**TABLE 4: MODEL COEFFICIENTS FOR 2<sup>3</sup>-FULL FACTORIAL DESIGN OBTAINED FROM LEAST SQUARE REGRESSION ANALYSIS**

Interactions	Model coefficients	LS			RM			$R_s$
		PA	$R_t$	$A_s$	PA	$R_t$	$A_s$	
Mean	$\beta_0$	942,746.54	6.91	1.27	612,026.78	10.06	1.43	7.71
$X_1$	$\beta_1$	-6840.02	0.60	-0.14	-7153.44	0.35	-0.07	-0.55
$X_2$	$\beta_2$	-25,874.18	-0.28	-0.13	24,378.96	-1.03	-0.16	-0.67
$X_3$	$\beta_3$	3257.99	0.84	0.05	-21,822.83	1.44	0.03	0.62
$X_1X_2$	$\beta_{12}$	-17,272.04	-0.05	0.11	23,177.15	-0.36	0.06	-1.21
$X_2X_3$	$\beta_{23}$	-22,204.34	-0.10	-0.06	-1508.07	-0.21	-0.04	0.02
$X_3X_1$	$\beta_{31}$	-17,404.39	0.21	-0.07	10,725.36	0.15	-0.02	-0.40
$X_1X_2X_3$	$\beta_{123}$	10,944.53	-0.04	0.07	-7907.94	-0.07	0.02	0.06

PA=Peak area,  $R_t$ =Retention time,  $A_s$ =Asymmetry factor,  $R_s$ =Resolution, LS=Losartan potassium, RM=Ramipril



**Fig. 4:** Three dimensional surface plots of responses predicted for losartan potassium and ramipril.

Three dimensional surface plots for losartan with predicted responses plotted on y-axis for (a) peak area (b) retention time (c) asymmetric factor and (d) resolution, and for ramipril with predicted responses plotted on y-axis for (e) peak area (f) retention time (g) asymmetric factor and (h) resolution are given in the figure. Predicted responses are obtained as a function of two factors, while the third factor was held at optimum level.

was found to have significant effect on retention time of LS, whereas pH has significant effect on retention time of RM as indicated in fig. 4b and f indicating sensitivity of retention time amongst studied factors. But, pH and % TFA were found to have no significant impact on retention time of LS and RM, respectively. Additionally, none of the factors studied showed significant impact on asymmetry factor for both the drugs as indicated in fig. 4c and g. Resolution showed sensitivity towards % AQ as increase in aqueous phase composition resulted in increased resolution (fig. 4d and h), however, the method demonstrated acceptable resolution at all studied levels of phase composition. The other studied factors pH and % TFA showed no significant impact on resolution, thus indicating the robustness of the

**TABLE 5: SYSTEM SUITABILITY PARAMETERS**

Peak parameter <sup>a</sup>	LS	RM
Capacity factor ( $K'$ )	1.90 ( $\pm 0.01$ )	2.98 ( $\pm 0.03$ )
Asymmetry factor ( $A_s$ )	1.17 ( $\pm 0.05$ )	1.29 ( $\pm 0.06$ )
Theoretical plates ( $N$ )	8250.96 ( $\pm 223.43$ )	8450.21 ( $\pm 277.28$ )
HETP	330.04 ( $\pm 8.94$ )	338.01 ( $\pm 11.09$ )
Resolution ( $R_s$ )	7.09 ( $\pm 0.12$ )	

LS=Losartan potassium, RM=Ramipril, HETP=Height equivalent to theoretical plates. <sup>a</sup> $n=6$  (three different sets of mixed standards were injected)

developed method. Even though few factors have shown effect on retention time of both the drugs, peak area was almost unaffected, which demonstrates robustness of the method.

Results obtained from system suitability test for both the drugs are presented in Table 5. The system



**TABLE 6: TEST SAMPLES (MARKETED AND IN-HOUSE TABLET FORMULATIONS) ANALYSIS**

Drug	Product	Amount recovered <sup>a</sup> (mg)	Recovery (%)	RSD (%)
LS	Marketed <sup>b</sup>	49.77	99.54	0.50
	In-house <sup>c</sup>	49.35	98.70	0.57
RM	Marketed <sup>b</sup>	4.96	99.20	0.50
	In-house <sup>c</sup>	4.93	98.60	0.61

RSD=Relative standard deviation. <sup>a</sup>n=3 (three different injections). <sup>b</sup>LORAM-5 (Kalindi Medicare Pvt. Ltd.) tablet containing 50 mg of losartan potassium (LS) and 5 mg of ramipril (RM). <sup>c</sup>In-house tablet formulation containing 50 mg of LS and 5 mg of RM

performance parameters obtained from system suitability test such as capacity factor ( $K' > 2$ ), tailing factor ( $T < 2$ ), number of theoretical plates ( $N > 2000$ ), and resolution ( $R_s > 2$ ) were found to be within limits for both the drugs indicated that the developed method is precise and stable for determination of LS and RM.

The drug peaks exhibited no chromatographic or response changes for 72 h at room temperature when compared against freshly prepared standards. The RSD values for LS and RM were found to be 1.85 and 2.09%, respectively. The results were within  $\pm 2.5\%$  at all studied time points indicating that the drugs were stable in mobile phase for 72 h at ambient temperature.

The proposed method was successfully employed for the determination of LS and RM in marketed and in-house prepared tablets. The mean drug content for LS and RM in both tablet preparations is shown in Table 6. The drug content obtained by analysis was found to be in good agreement with labelled claim of both the products. The method was found to be accurate with mean absolute recoveries more than 98% for both of the drugs. Moreover, the formulation excipients did not show significant interference in determination of either of the drugs as % bias values were  $< 0.61\%$  for either of the drugs. Overall, the method was found to be accurate and selective for the determination of LS and RM in both tablet formulations.

In summary, a simple, sensitive, accurate and precise method was developed for the quantitative determination of LS and RM in bulk and pharmaceutical formulations. Novel microwave-assisted forced degradation studies were done to evaluate the selectivity of the method. Force degradation studies confirmed the selectivity of the method for stability analysis of products containing LS and RM, in combination. The developed method

was successfully validated for selectivity, linearity and range, accuracy, precision, sensitivity. DoE technique was successfully applied for establishing robustness of the method. This method can be used for routine analysis of samples and tablet formulations containing LS and RM.

## REFERENCES

- Joshi SR, Yeolekar ME, Tripathi KK, Giri J, Maity AK, Chopda M, *et al.* Evaluation of efficacy and tolerability of losartan and ramipril combination in the management of hypertensive patients with associated diabetes mellitus in India (LORD Trial). *J Assoc Physicians India* 2004;52:189-95.
- Polinko M, Riffel K, Song H, Lo MW. Simultaneous determination of losartan and EXP3174 in human plasma and urine utilizing liquid chromatography/tandem mass spectrometry. *J Pharm Biomed Anal* 2003;33:73-84.
- González L, López JA, Alonso RM, Jiménez RM. Fast screening method for the determination of angiotensin II receptor antagonists in human plasma by high-performance liquid chromatography with fluorimetric detection. *J Chromatogr A* 2002;949:49-60.
- Iwasa T, Takano T, Hara K, Kamei T. Method for the simultaneous determination of losartan and its major metabolite, EXP-3174, in human plasma by liquid chromatography-electrospray ionization tandem mass spectrometry. *J Chromatogr B Biomed Sci Appl* 1999;734:325-30.
- Soldner A, Spahn-Langguth H, Mutschler E. HPLC assays to simultaneously determine the angiotensin-AT1 antagonist losartan as well as its main and active metabolite EXP 3174 in biological material of humans and rats. *J Pharm Biomed Anal* 1998;16:863-73.
- Ritter MA, Furtek CI, Lo MW. An improved method for the simultaneous determination of losartan and its major metabolite, EXP3174, in human plasma and urine by high-performance liquid chromatography with fluorescence detection. *J Pharm Biomed Anal* 1997;15:1021-9.
- Furtek CI, Lo MW. Simultaneous determination of a novel angiotensin II receptor blocking agent, losartan, and its metabolite in human plasma and urine by high-performance liquid chromatography. *J Chromatogr* 1992;573:295-301.
- Gowda KV, Mandal U, Senthamil Selvan P, Sam Solomon WD, Ghosh A, Sarkar AK, *et al.* Liquid chromatography tandem mass spectrometry method for simultaneous determination of metoprolol tartrate and ramipril in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* 2007;858:13-21.
- Persson BA, Fakt C, Ervik M, Ahnoff M. Interference from a glucuronide metabolite in the determination of ramipril and ramiprilat in human plasma and urine by gas chromatography-mass spectrometry. *J Pharm Biomed Anal* 2006;40:794-8.
- Lu XY, Shen-Tu JZ, Liu J. High-performance liquid chromatography-mass spectrometric analysis of ramipril and its active metabolite ramiprilat in human serum: Application to a pharmacokinetic study in the Chinese volunteers. *J Pharm Biomed Anal* 2006;40:478-83.
- Zhu Z, Vachareau A, Neirinck L. Liquid chromatography-mass spectrometry method for determination of ramipril and its active metabolite ramiprilat in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* 2002;779:297-306.
- Hertzog DL, McCafferty JF, Fang X, Tyrrell RJ, Reed RA. Development and validation of a stability-indicating HPLC method for the simultaneous determination of losartan potassium, hydrochlorothiazide, and their degradation products. *J Pharm Biomed Anal* 2002;30:747-60.
- Erk N. Analysis of binary mixtures of losartan potassium and hydrochlorothiazide by using high performance liquid chromatography, ratio derivative spectrophotometric and compensation technique. *J Pharm Biomed Anal* 2001;24:603-11.

14. Carlucci G, Palumbo G, Mazzeo P, Quaglia MG. Simultaneous determination of losartan and hydrochlorothiazide in tablets by high-performance liquid chromatography. *J Pharm Biomed Anal* 2000;23:185-9.
15. Hogan BL, Williams M, Idiculla A, Veysoglu T, Parente E. Development and validation of a liquid chromatographic method for the determination of the related substances of ramipril in Altace capsules. *J Pharm Biomed Anal* 2000;23:637-51.
16. Bonazzi D, Gotti R, Andrisano V, Cavrini V. Analysis of ACE inhibitors in pharmaceutical dosage forms by derivative UV spectroscopy and liquid chromatography (HPLC). *J Pharm Biomed Anal* 1997;16:431-8.
17. Belal F, Al-Zaagi IA, Gadkariem EA, Abounassif MA. A stability-indicating LC method for the simultaneous determination of ramipril and hydrochlorothiazide in dosage forms. *J Pharm Biomed Anal* 2001;24:335-42.
18. Zhao Z, Wang Q, Tsai EW, Qin XZ, Ip D. Identification of losartan degradates in stressed tablets by LC-MS and LC-MS/MS. *J Pharm Biomed Anal* 1999;20:129-36.
19. Williams RC, Alasandro MS, Fasone VL, Boucher RJ, Edwards JF. Comparison of liquid chromatography, capillary electrophoresis and super-critical fluid chromatography in the determination of losartan potassium drug substance in Cozaar tablets. *J Pharm Biomed Anal* 1996;14:1539-46.
20. McCarthy KE, Wang Q, Tsai EW, Gilbert RE, Dominic IP, Brooks MA. Determination of losartan and its degradants in COZAAR® tablets by reversed-phase high-performance thin-layer chromatography. *J Pharm Biomed Anal* 1998;17:671-7.
21. Baing MM, Vaidya VV, Sane RT, Menon SN, Dalvi K. Simultaneous RP-LC determination of losartan potassium, ramipril and hydrochlorothiazide in pharmaceutical preparations. *Chromatographia* 2006;64:293-6.
22. Bende G, Kollipara S, Kolachina V, Saha R. Development and validation of a stability indicating RP-LC method for determination of imatinib mesylate. *Chromatographia* 2007;66:859-66.
23. ICH. Q2B, Harmonised Tripartite Guideline, Validation of Analytical Procedure: Methodology, IFPMA. In: Proceedings of the International Conference on Harmonization, Geneva, March 1996.
24. United States Pharmacopoeia. Validation of Compendial Methods. 26th ed. Maryland: Pharmacopoeia Convention Inc.; 2003. p. 2439-42.
25. Pellett J, Lukulay P, Mao Y, Bowen W, Reed R, Ma M, *et al.* "Orthogonal" separations for reversed-phase liquid chromatography. *J Chromatogr A* 2006;1101:122-35.
26. Bolton S. Analysis of Variance. *Pharmaceutical Statistics: Practical and Clinical Applications*. New York, USA: Marcel Dekker; 1997. p. 182-221.

**Accepted April 28, 2012**

**Revised April 17, 2012**

**Received May 27, 2011**

**Indian J. Pharm. Sci., 2012, 74 (3): 201-210**