HPLC-PDA Determination of Losartan Potassium and Hydrochlorothiazide Using Design of Experiments

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ABSTRACT

A simple, rapid, precise, accurate, sensitive and stability indicating high performance liquid chromatographic method has been developed for simultaneous determination of losartan potassium and hydrochlorothiazide in pharmaceutical formulations. Method development and optimization was carried out using 'Design of Experiments' (DoE). Optimized mobile phase (v/v/v) was water (containing 0.25 ml/L triethylamine), methanol and acetonitrile (60:38:30, pH adjusted to 2.7±0.1). Chromatographic separation was achieved on Hypersil®-Gold C18 (150 x 4.6 mm, 5 µm, Thermo Fisher Scientific, USA), column at 25 ± 2°C. The mobile phase flow rate was 1.0 m/min. The analysis was carried out at 271 nm using 10 µl samples. The method was validated as per the International Conference on Harmonization (ICH) guidelines. This method had a chromatographic run time of 10.0 min and a linear calibration curves ranged from 2.0 to 48.0 µg/mL for losartan potassium, and 0.5–12.0 µg/mL for hydrochlorothiazide. The limits of detection for hydrochlorothiazide and losartan potassium were 0.013 and 0.138 µg/ml while the limits of quantitations were 0.039 and 0.418 µg/ml, respectively. Stability studies indicate that the degradation of drugs was higher during oxidative stress than other stress. No photolytic degradation was observed

Keywords: HPLC, Losartan Potassium, hydrochlorothiazide, Box-Behnken Design, Design of experiments (DoE), Response surface methodology (RSM).

1. INTRODUCTION

Losartan potassium (LoP, Angiotensin II receptor antagonist) is chemically potassium 5-(4'-{[2-butyl-4-chloro-5- (hydroxymethyl)- 1*H* - imidazol- 1- yl] methyl} biphenyl-2-yl)tetrazol-1-ide (Figure 1a). Angiotensin II receptor antagonists, also known as angiotensin receptor blockers (**ARBs**), AT_1-receptor antagonists or sartans, are a group of pharmaceuticals that modulate the renin-angiotensin-aldosterone system. ARBs (LoP, valsartan, candisartan, irbesartan, olmisartan) are better in preventing first occurrence of atrial fibrillation than beta-blocker (atenolol) or calcium antagonist (amlodipine) therapy. LoP is a strong non-peptide antihypertensive agent and has a gradual, long-lasting effect as an antihypertensive. Hydrochlorothiazide (HCT), 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide is used as a diuretic (Figure 1b). HCT is used along with different cardiovascular agent in the management of hypertension [1-4].

Recently several methods have been reported for the simultaneous determination of HCT, LoP and other drugs, including HPTLC[5], supercritical fluid chromatography[6], Capillary electrophoresis[7], capillary electrochromatography[8], spectroscopy[9-11] and HPLC[12–21]. In biological fluids, the active principles have been determined simultaneously by LC-MS [22,23]. These methods are not simple to perform; they are time consuming, which require careful and tedious sample

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preparation due to the different acid-base characteristics of the analytes, and use of expensive instruments that are usually not available in all quality control laboratories. Literature survey reveals that a non-stability indicating HPLC method [24] was developed utilizing chemometric protocol. International Conference on Harmonization (ICH) guidelines requires performance of stress tests on the drug substance, which can help to identify the likely degradation products. Moreover, validated stability-indicating methods should be applied in the stability studies once they have demonstrated their suitability for their intended purpose [25,26]. The aim of the present work is to develop and validate an UFLC stability-indicating method for determining HCT and LoP in its pharmaceutical dosage forms as per ICH guideline. Stress studies are performed in order to validate the stability-indicating power of the developed analytical method and to identify the key factors that will impact the stability of the drug. Hence the present method is developed using design of experiment utilizing Box-Behnken design [27].

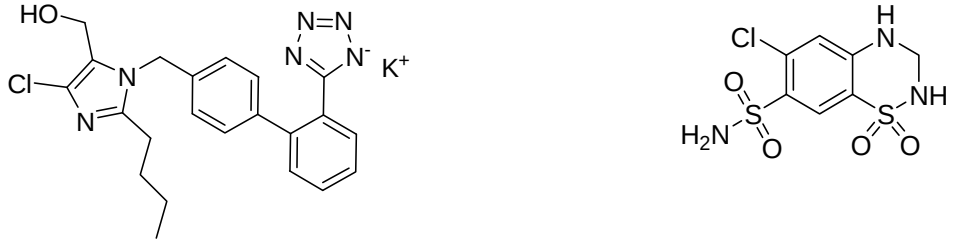


Figure 1. Structure of (a) Losartan Potassium and (b) Hydrochlorothiazide

EXPERIMENTAL

Chemical and reagents

Losartan potassium (LoP) and hydrochlorothiazide (HCT) were obtained as a gift sample from Hetero Pharmaceutical Ltd, Hyderabad, India. Formulation of LoP and HCT (Losar*-H) were purchased from local pharmacies in India. Acetonitrile and methanol (HPLC grade) were purchased from Tedia Company (Fairfield, USA). Double distilled and deionised water was used throughout the analysis.

Chromatographic system and conditions

A Prominence UFLC system, consisted of a 20-AD UFLC pump, DGU-20A_3 degasser, SIL-20A auto-sampler, SPD-M20A photo diode array detector and a CBM-20A communication bus module (Shimadzu, Kyoto, Japan) was used. The signals were captured using LC-solution version 1.25 (2009-2010) work station (Shimadzu, Japan) operating under Microsoft Windows 7 (32 bit) platform. The pH measurements were carried out using calibrated WTW 720 pH meter equipped with a combined electrode (with ATC probe). Chromatographic separation was achieved on Hypersil®-Gold C_{18} (150 x 4.6 mm, 5 µm, Thermo-Fisher Scientific, USA) column, at 25 °C, using optimised mobile phase *(v/v/v)* consisting of water (containing triethylamine 0.25 ml/L), methanol and acetonitrile (60:38:30, pH 2.7 ± 0.1). The mobile phase flow rate was 1.0 ml/min. The analysis was performed at 271 nm, while the signals were captured from 200 to 350 nm using 10 µl samples.

Preparation of stock solutions, calibration standards and quality control samples

Stock solution (1 mg/ml) of LoP and HCT were prepared separately in aqueous methanol (50%) and stored in refrigerator. Working solutions, calibration standards (0.5-12 µg/ml for HCT and 2-48 µg/ml for LoP) and quality control samples (HCT- 0.5, 5 and 12 µg/ml; LoP- 2, 20 and 48 µg/ml) were prepared separately as required and stored in amber colored glass-wares in refrigerator.

Software aided method optimization

Response surface methodology

Response surface methodology utilises "Central

composite design" and "Box-Behnken design" for method development and optimization. Selection of mobile phase is an important requirement in method development. Various factors were considered for method development including, volume fraction of aqueous and organic solvents in mobile phase. In order to determine the optimal condition a Box-Behnken design (BBD) was selected as it required only 17 runs with 3 variables. In this design the extreme factors does not affect the analysis. Seventeen experiments (with three variables) were designed and constructed. The conditions, observed responses and levels described in Table 1-2. Design Expert® software (Version 8.0.6, Stat-Ease Inc., Minneapolis, MN) was used for experimental design, data analysis and construction of regression model.

$$Y = b_o + b_1A + b_2B + b_3C + b_4AB + b_5AC + b_6BC + b_7A^2 + b_8B^2 + b_9C^2 \quad ...(eq.\ 1)$$

Where *Y* is the measured response (resolution factor Rs, or retaintability T_p/R_t) associated with each factor level combination: Water (*A*), Methanol (*B*) and Acetonitrile (*C*). The minimum and maximum volume fractions for aqueous phase were 50 and 70, respectively. The minimum and maximum volume fractions for organic phase were selected as 20 and 40, respectively.

Mobile phases of different composition (*v/v/v*) were prepared and the pH of the mobile phase was adjusted to 2.7 ± 0.1 (using 25% orthophosphoric acid). Samples were analysed separately and as a mixture. Retention times, ultraviolet spectrum and peak purity were used to identify different drugs. The resolution factor (R_s) and retaintability (theoretical plates T_p/retention time R_t) were calculated using LC-solution chromatographic software and are presented in Table 1.

Table 1. Coded values for factor level and observed responses in Box-Behnken design for 17 analytical trial

Exp. (Run)	Std Run	Type	Water A (%)	Methanol B (%)	Acetonitrile C (%)	Resolution factor $Y_1=R_s$	Retaintability (HCT) $Y_2=T_p/R_t$	Retaintability (LoP) $Y_3=T_p/R_t$
1	9	IBFact	0	-1	-1	27.4	1210	890
2	13	Center	0	0	0	14.0	1279	1305
3	6	IBFact	1	0	-1	23.0	1070	690
4	2	IBFact	1	-1	0	17.1	1180	1130
5	17	Center	0	0	0	14.5	1400	1340
6	16	Center	0	0	0	13.8	1350	1310
7	10	IBFact	0	1	-1	16.7	915	585
8	4	IBFact	1	1	0	11.5	1090	1065
9	5	IBFact	-1	0	-1	18.0	1200	1055
10	15	Center	0	0	0	14.1	1300	1375
11	11	IBFact	0	-1	1	9.3	1265	1285
12	14	Center	0	0	0	13.5	1250	1350
13	3	IBFact	-1	1	0	6.7	1105	1210
14	7	IBFact	-1	0	1	5.8	1325	1455
15	1	IBFact	-1	-1	0	13.5	1350	1370
16	12	IBFact	0	1	1	8.9	1195	1315
17	8	IBFact	1	0	1	10.1	1265	1420

Data analysis [27]

Analysis of variance (ANOVA), a regression analysis and the plotting of response surface were performed to establish optimum conditions for the separation and retaintability of analytes. ANOVA was used to test adequacy and fitness of the responses for linear, 2 function interaction (2FI) and quadratic functions of the variables. A model with P-values (P > F) less than 0.05 was regarded as significant. The lack-of-fit test was used to compare the residual and pure errors at the replicated design points. Lack of fit is measuring how well the model fits the data. Strong lack of fit (P < 0.05) is an undesirable property, because it indicates that the model doesn't fit the data well. It is desirable to have an insignificant lack of fit (P > 0.1). The highest-order significant polynomial with not significant lack of fit was selected. The predicted residual sum of the squares (PRESS) was used as a measure of fit of the model to the points in the design. After predicting the optimal conditions for separation, the experiment was repeated to ensure the reliability of the predicted values and experimental data.

Table 2. Experimental factors and level used in the Box-Behnken design

Factor	Low (-1)	Medium (0)	High (+1)
Independent			
A=Water,	50	60	70
B= Methanol	20	30	40
C= Acetonitrile	20	30	40
Dependent			
$Y_1 = R_s$ = Resolution Factor (HCT and Losartan)			
$Y_2 = T_p/R_t$ = Retaintability of HCT			
$Y_3 = T_p/R_t$ =Retaintability of Losartan			

Preparation of mobile phase

The measured amount of water (600 ml, containing 0.25 ml/L TEA), methanol (380 ml each) and acetonitrile (300 ml) were transferred in HPLC bottle separately. The final pH was adjusted to 2.7 ± 0.1, using 25% orthophosphoric acid. Mobile phase was filtered through 0.22 µm nylon filters and degassed using ultrasonic bath (Branson, Model 3210, USA).

Preparation of Sample for Assay

Average weight of twenty tablets (containing 12.5 mg HCT and 50 mg LoP) was determined. Tablets were triturated to a fine powder. A quantity of powder equivalent to 12.5 mg HCT and 50 mg LoP was weighed accurately and transferred to 100 ml calibrated volumetric flask. Aqueous methanol (50%, 25mL) was added and contents were sonicated for 10 minutes. The volume was made up to the mark. The solution was filtered through 0.45 µm nylon filter (Micro-syringe filter). Appropriate dilutions (in the calibration range) were prepared for analysis.

Analytical Method validation

The analytical method was validated for accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), specificity, robustness and ruggedness as per recommendation of International Conference on Harmonisation (International Conference on Harmonisation, 2005).

Linearity, LOD, and LOQ

Calibration standards (HCT 0.5-12 µg/ml and LoP 2-48 µg/ml) were prepared using aliquots of stock solution (1000 µg/ml) or working solutions (50, 100, 500 µg/ml)

of HCT and LoP. Replicates of each concentration were independently prepared and injected into the chromatograph. Linearity was evaluated by the linear least-squares regression model. Microsoft office excel 2007 was used for statistical analysis. The method was validated according to ICH guidelines. A 5% significance level was used for evaluation. LOD and LOQ were calculated as $3.3\times\sigma_{n-1}/S$ and $10\times\sigma_{n-1}/S$, where σ_{n-1} is the standard deviation of the intercept and S is the slope of the calibration curve.

Table 3. Regressed equation obtained for the resolution factor and retaintability of analytes

Models	SD	R^2	Adjusted R^2	Predicted R^2	PRESS[#]
Resolution Factor (Y_1)					
Linear	1.98	0.8946	0.8703	0.7805	106.2934
2FI	1.55	0.9504	0.9206	0.7497	121.2441
Quadratic	***0.36***	***0.9981***	***0.9956***	***0.9853***	***7.1363***
Cubic	0.37	0.9989	0.9955	Aliased	*
Regression equation of the fitted quadratic model for Y_1 after model reduction					
$Y_1 = R_s = 11.1677 + 2.1128.A - 1.066.B - 2.489.C + 0.0257B.C - 0.0157\ A^2 + 0.0179\ C^2$					
Retaintability for HCT (Y_2)					
Linear	91.13	0.5509	0.4473	0.2989	168557.41
2FI	93.85	0.6336	0.4138	0.1111	213718.81
Quadratic	***45.26***	***0.9404***	***0.8637***	***0.8982***	***24476.25***
Cubic	59.57	0.9410	0.7639	Aliased	*
Regression equation of the fitted quadratic model for Y_2 after model reduction					
$Y_2=T_p/R_t = 549.20395 - 4.687A + 36.403B + 33.09C + 0.5625B.C - 1.0338B^2 - 0.6963C^2$					
Retaintability for LoP (Y_3)					
Linear	149.28	0.7197	0.6550	0.5224	493689.55
2FI	152.37	0.7754	0.6406	0.2994	724125.49
Quadratic	***23.03***	***0.9964***	***0.9918***	***0.9896***	***10765.62***
Cubic	29.03	0.9967	0.9870	Aliased	*
Regression equation of the fitted quadratic model for Y_3 after model reduction					
$Y_3=T_p/R_t = 646.02-34.5625A + 52.269B + 60.457C + 0.825AC + 0.8375\ BC - 1.394B^2-1.7815C^2$					

** Case(s) with leverage of 1.0000; [#]predicted residual sum of the squares (PRESS) statistics not defined.*

System Suitability

System suitability was evaluated using data analysis of six replicate injections of a sample solutions (8.0 µg/ml HCT and 32.0µg/ml LoP) according to "USP"[28]. Chromatographic parameters like retention time, area (AUC), peak height (AU), tailing factor, theoretical plate, USP width and height equivalent to theoretical plates (HETP) were studied and the precision (RSD) was

evaluated. The precision, as measured by coefficient of variation was determined at each set parameters and it should be less than 2%.

Precision

Quality control samples (n=3) were analysed on different occasions (n=3) and three different days. The precision (RSD) of the method was determined as intraday precision (repeatability) and intermediate precision. The intermediate precision was estimated from the RSD of the analysis of the samples prepared at the same concentration but on 3 different days at different concentration levels, while intraday precision was calculated by analyzing the same concentration during the same day at different time.

Table 4. Linearity data of the proposed method

HCT		**LoP**	
Concentration (µg/ml)	Mean Area	Concentration (µg/ml)	Mean Area
0.5	20,325	2	14,007
1	40367	4	28,069
2	80285	8	56,079
4	160578	16	112,612
6	240694	24	166,657
8	320418	32	220,656
10	399631	40	277,522
12	480416	48	332,521
Slope	39,980	**Slope**	6,913
Intercept	446.3	**Intercept**	664.5
r^2	0.99990	**r^2**	0.99996
y=39,980.3(±80.4)x + 446.3(±372.2)		*y=6,912.7(±26.8)x + 664.4(±203.7)*	

Table 5. Recovery study (Accuracy of the method)

Drug	**Amount taken (mg)**	**Amount Added**		**Amount recovered (mg)**	**%**	**SD**	**RSD**
		%	**mg**				
HCT	12.5	80	10.0	22.47	99.9	0.07	0.32
	12.5	100	12.5	25.19	100.8	0.07	0.27
	12.5	120	15.0	27.55	100.2	0.10	0.36
LoP	50.0	80	40.0	89.65	99.6	0.98	1.09
	50.0	100	50.0	100.29	100.3	0.19	0.19
	50.0	120	60.0	111.03	100.9	0.94	0.86

Accuracy

Accuracy (as percentage recovery) was measured using replicate sample of analytes prepared using tablet triturate assayed earlier. Different samples (at level 80%,

100% and 120%) were prepared using tablet triturate (12.5 mg HCT and 50 mg LoP as 100 %) and adding known quantity of HCT and LoP (at 80% - 120% level). From these fortified samples, appropriate sample solutions were prepared, analyzed and the total amount recovered was calculated. Accuracy was calculated by comparing with true value. The concentrations were back calculated by regression equations.

Table 6. Precision study of the proposed method

Drug	Concentration (µg/ml)	Intraday precision (n=3)		Interday precision (n=3)	
		Area Count (Mean ± SD)	RSD	Area Count (Mean ± SD)	RSD
HCT	0.5	20263.9 ± 294.0	1.45	20044.8 ± 357.4	1.78
	5.0	199429.1 ± 864.7	0.43	199270.4 ± 501.0	0.25
	12.0	479659.5 ± 789.9	0.17	481724.6 ± 1228.4	0.26
LoP	2.0	14154.8 ± 138.0	0.97	14096.3±145.4	1.03
	20.0	83666.2 ± 756.2	0.90	84376.9±968.6	1.15
	48.0	333979.7 ± 1062.1	0.32	336355.2±4507.8	1.34

Table 7. System suitability parameters for HCT and LoP

Parameter	T_r (min.)	Area	Height	Tailing Factor	Theoretical Plate	USP Width	HETP
HCT							
Mean	2.071	319899.8	68348.3	1.288	3508.50	0.141	28.529
SD	0.000	78.70	1.03	0.003	3.94	0.001	0.001
RSD	0.02	0.02	0.00	0.20	0.11	0.39	0.00
LoP							
Mean	5.581	221591.2	23776.7	1.062	7301.83	0.261	13.576
SD	0.001	5.845	0.816	0.002	68.52	0.001	0.036
RSD	0.01	0.00	0.00	0.22	0.94	0.21	0.27

Ruggedness

Ruggedness was accessed by intentionally changing the chromatographic parameters (wavelength or flow rate) and evaluating their impact on analysis. The ruggedness of the method was evaluated on the basis of precision (RSD < 2%).

Stability Studies

Stress studies like oxidative, alkaline, acidic stress, exposure to sunlight and UV light (254 nm), were conducted using raw material and tablets. Samples were exposed to different stressed conditions, processed and analyzed (n=3). The chromatograms of the samples were compared with those of control samples that were freshly prepared from the stock solution and without stress. All samples were analyzed in triplicate. The peak purity was checked using the chromatographic tools of the LC-Solution software. This assessment was based on the

comparison of spectra recorded during the elution of the peak. UV spectra and peak purity were used to assess purity of analyte.

For evaluation of oxidative stress, HCT (5 mg) and LoP (20 mg) were weighed accurately and transferred to 50 ml volumetric flask. Five ml hydrogen peroxide (25%) was added, and content were shaken for an hour. The contents were diluted to 50 ml with mobile phasE. Similarly for evaluation of acidic or alkaline stress, analytes (HCT 5 mg and LoP 20 mg) were weighed accurately and transferred to 50 ml volumetric flask. These samples were shaken with 5 ml of either 1M hydrochloric acid (HCl) or 1M sodium hydroxide (NaOH) for 1 h. After one hour the content were diluted to 50 ml with mobile phase.

A physical mixture of HCT and LoP (1g, 20:80, *w/w*) was placed in an open watch glass and exposed to either UV-irradiation (~100 W/m^2) or direct sunlight for two hours with occasionally shifting of the content using stainless steel spatula. After 2 hours, 25 mg of sample was weighed, transferred to 50 ml volumetric flask and dissolved in mobile phase. All the solutions were suitably diluted and analyzed.

Stress studies were also conducted on tablet formulation. Tablet triturate (powder) equivalent to 5mg of HCT and 20 mg of LoP was used in stress testing. Samples were processed and analyzed as described above.

RESULTS AND DISCUSSION

Analytical Method Development

In order to achieve optimum separation various parameters like solvent, solvent strength, detection wavelength, flow rate, elution time, asymmetry, and theoretical plate numbers were considered. During method development the column was saturated for at-least 1 hour with different mobile phases prior to analysis. Analytes were analysed using 17 different mobile phases (as per Box-Behnken Design protocol) and the retention time, tailing factor, retaintability along with resolution factor was calculated. HCT and LoP were eluted at ~2.0 and 5.4 min using optimised mobile phase. Although temperature was found not to be a critical parameter for this analysis, it was set at 25 ± 2°C. The absorption maximum of the drug at 271 nm was selected for detection, as there was no interference from excipients present in drug. The resolution factor was ~10.75. Figure 2a-b depicts the representative chromatogram obtained using present method.

Table 8. Stability data under different stressed conditions

Stress condition	API		Tablets	
	% HCT remained	% LoP Remained	% HCT remained	% LoP remained
Oxidative Stress (25% H_2O_2)	96.72	60.85	99.93	99.20
Acidic Stress (1N HCl)	98.51	99.51	99.95	99.95
Alkaline stress (1N NaOH)	99.85	99.65	100.0	99.90
Ultraviolet light (2 Hour, 80$W.m^{-2}$)	99.90	99.91	100.0	99.95
Direct Sunlight	100.0	99.98	100.0	100.0
Aqueous Stability (After 15 days)	99.90	99.83	99.86	99.80

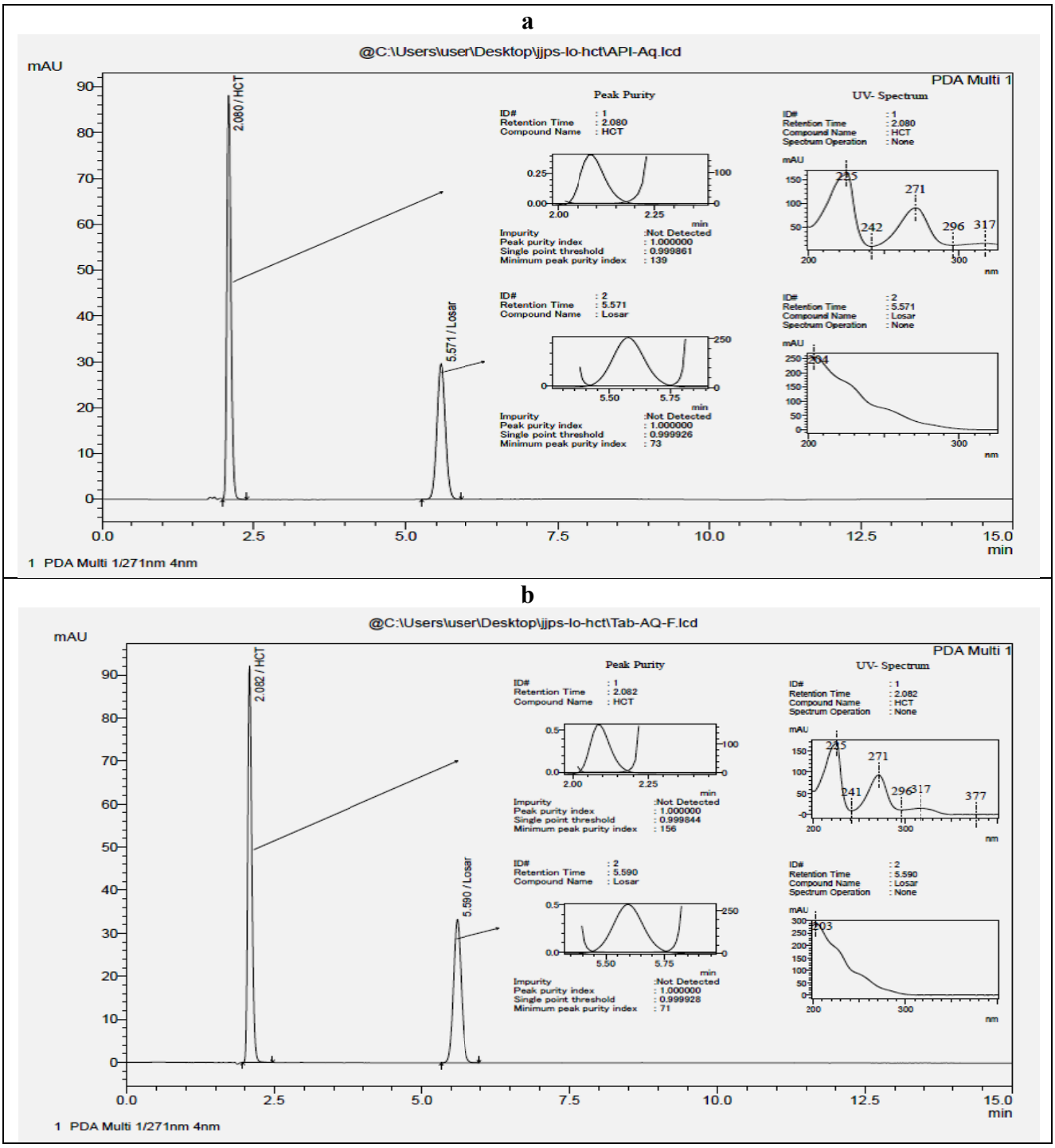
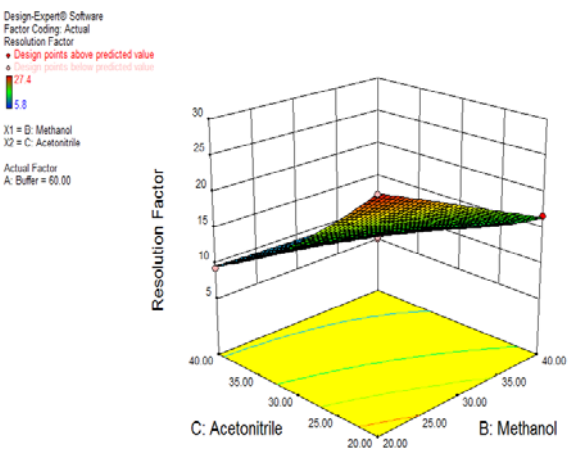


Figure 2. Chromatogram, Peak purity (inset) and UV spectra (inset) of analytes (HCT and LoP), (a) fresh solution of API and (b) extracted solution from tablets

A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Resolution Factor
Design points above predicted value
Design points below predicted value
27.4
5.8
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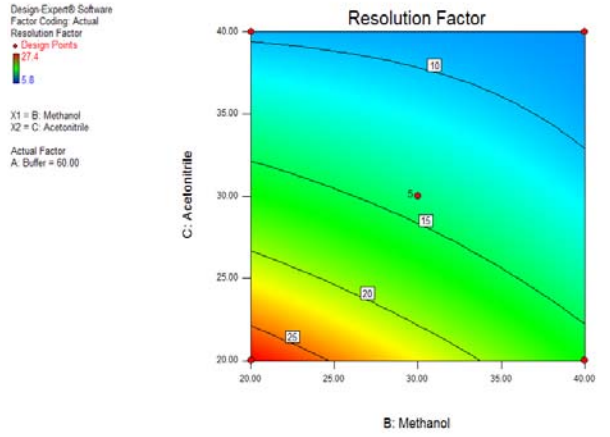
X2 = C: Acetonitrile
Actual Factor
A: Buffer = 60.00
Resolution Factor
C: Acetonitrile
B: Methanol

B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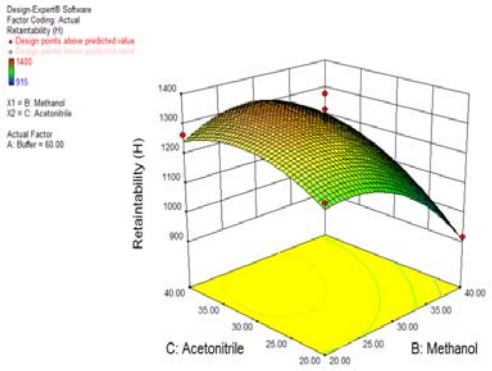
B: Methanol

C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Retaintability (H)
Design points above predicted value
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915
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Actual Factor
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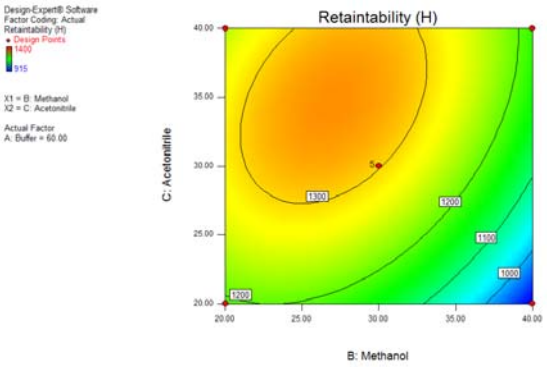
Retaintability (H)
C: Acetonitrile
B: Methanol

D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C: Acetonitrile
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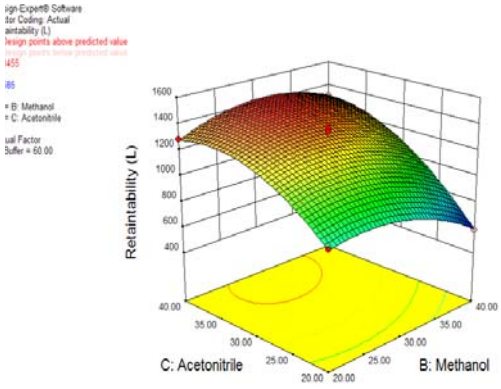
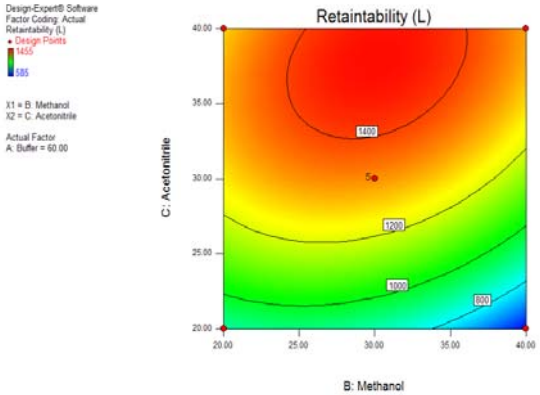
E


Retaintability (L)
C: Acetonitrile
B: Methanol

F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Design Points
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C: Acetonitrile
B: Methanol

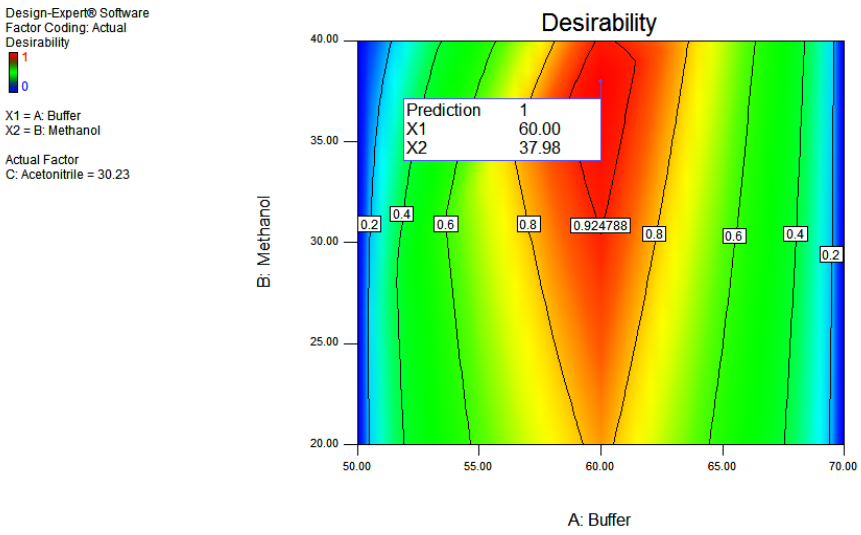

Figure 3. 3-D surface and 2-D contour plots for (a, b,) resolution factor; (c, d) retaintability factor for HCT (e, f) retaintability factor for LoP versus independent factor (A-buffer, B-methanol, C-acetonitrile); and (g) Point prediction utilizing desirability plot (for R_s=11).

Design of experiment and method optimization

A three factorial, Box-Behnken design was performed using 17 experimental runs. The independent, dependent variables and observed responses for all 17 trial experiments are given in Table 1-2. It was observed that the best fitted model was the quadratic model for different responses. The comparative values of *R* and *SD* for different proposed models are given in Table 3, along with regression equation generated for the finally selected response. Only statistically significant ($p < 0.05$) coefficient are included in the regressed equation after model reduction (insignificant model terms are removed to improve the model). A positive value in the equation indicates the favourable response while a negative value indicates an inverse relationship between the factor and the response. It is clear from the equations that the factor buffer (A) has a positive effect on the resolution factor, while acetonitrile (B) and methanol (C) are having negative effect. In case of retaintability these factors are producing opposite effect (Table 3).

Two dimensional contour plot and 3D response surface plots are presented as Fig. 3 (a-f), which are very useful for studying the interaction effects of independent factors on the responses. The coefficients for the model were estimated by least squares regression. The relationship between the response factors (R_s and retaintability) and independent factors is quadratic. An independent factor can produce different degree of response when the different factors (A, B or C) are changed simultaneously. Interaction of B and C, as well as A and C produce positive impact on resolution factor or retaintability). The square of factor A^2 is having negative impact on resolution of HCT and LoP; while B^2 and C^2 are having negative impact on retaintability factor (T_p/R_t) (Table 3).

As observed, an increase in buffer concentration at constant methanol and acetonitrile content increases the resolution factor (R_s). It is evident from the steepness of the curve that at constant buffer and methanol content, acetonitrile has significant influence on the resolution factor R_s (Fig. 3a, b). Keeping these observations in knowledge the resolution factor R_s was considered during method optimization and mobile phase selection step. It is evident from Fig. 3c-f, that the interaction of methanol (B) and acetonitrile (C), BC is having positive impact on retaintability. An increase in buffer content at constant

methanol (B=30) and acetonitrile (C=30) content decreases the retaintability of HCT and LoP.

The final composition of the mobile phase for simultaneous determination of these compounds was selected using Design Expert® software (Version 8.0.6, Stat-Ease, USA) after studying the 2D contour and 3D response surface plots. Resolution factor R_s was kept constant to 11.0 during the optimization step. The desirability plot (Fig. 3g) and several different mobile phase compositions were generated by the software. Using desirability plot, the optimized mobile phase (having desirability factor 1.000) was selected for analysis. Different drug combinations were analysed and the resolution factor was calculated. The observed resolution factor (R_s) was 10.75, using optimized chromatographic conditions. The results indicate that the present method is capable of separating HCT and LoP with significant retaintability. The developed and optimized mobile phase exhibit good resolution and reproducible results. The present method is rapid and stability indicating as compared to the method developed using chemometric protocol[24]. The typical chromatogram obtained during the analysis are given in Fig. 2a-b.

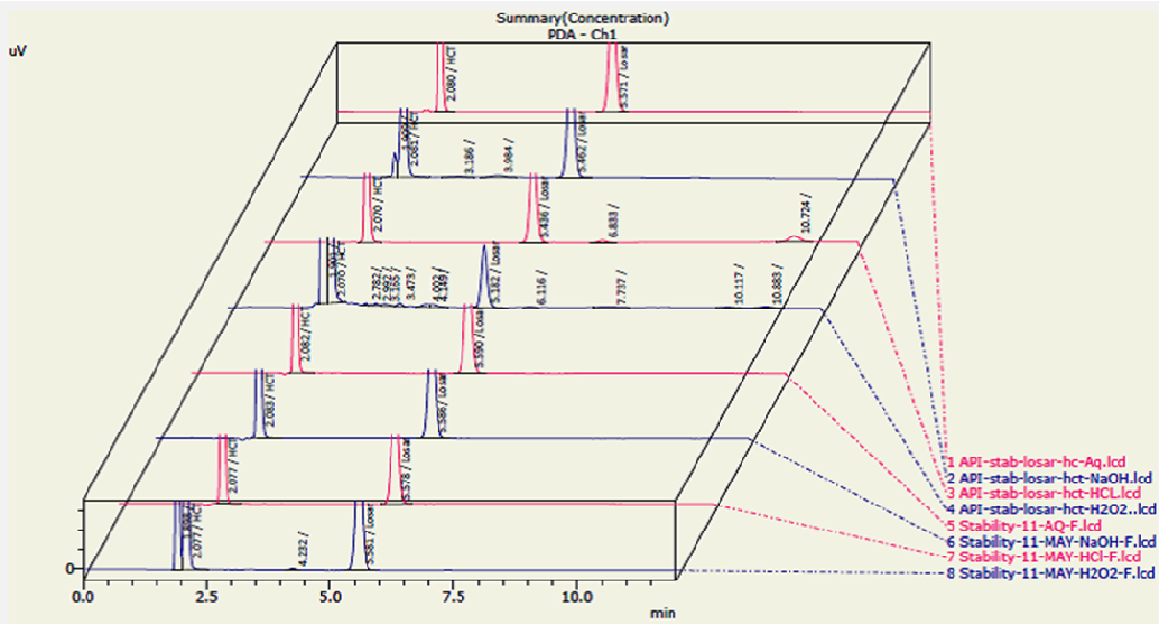

Figure 4. Chromatograph of analytes (HCT and LoP), (1) fresh solution of API, (2) API exposed to alkaline stress, (3) acidic stress, (4) oxidative stress, (5) Fresh sample extracted from tablets, (6) tablet exposed to alkaline stress, (7) acidic stress, (8) oxidative stress

Method Validation

The method was validated in respect to parameters like linearity, limit of quantitation (LOQ), limit of detection (LOD), precision, accuracy, specificity, robustness, system suitability and stability.

Linearity, LOD and LOQ

Calibration curves (n=5) were linear over the concentration range of 0.5-12 µg/ml and 2-48 µg/ml for HCT and LoP respectively. The mean regression equations for HCT and LoP were *y = 39,980.3(±80.4)x + 446.3(±372.2) and y = 6,912.7(±26.8)x + 664.5(±203.7)*

respectively ($r^2 \geq 0.9999$), using weighting factor-*x* (Table 4). The LOD and LOQ for HCT were 0.013 and 0.039 µg/ml, respectively. While for LOP 0.138 and 0.418 µg/ml respectively.

Accuracy and Precision

The accuracy and precision of the analytical method were established across its linear range as indicated in the guideline. Table 5 reveals that excellent recoveries were made at different concentration levels (80%, 100% and 120%) using present method. The percent recoveries were ranged from 99.6-100.9% and 99.9-100.8% for LoP and HCT respectively (Table 5). The intra-day precision and inter-day precision were less than 1.45 % and 1.78 % respectively (Table 6). Low precision values indicate the repeatability of the developed analytical method.

Specificity

Specificity of the method was assessed by comparing the chromatograms obtained from raw material and extracted drugs from formulation. The retention times of drug from standard solutions and formulation were identical and no co-eluting peaks or impurity from the diluents were observed, indicating specific method for quantitative estimation of drug in the commercial formulation (Figure 2 and inset)

System Suitability

System suitability parameters were studied with six replicates standard solution of the drugs and the calculated parameters are within the acceptance criteria. The tailing factor, the number of theoretical plates and HETP were in the acceptable limits (RSD less than 2%). The system suitability results are shown in Table 7.

Robustness

Robustness of the methods was illustrated by getting the resolution factor and tailing factor, when mobile phase flow rate (± 0.1 ml/min) and wave-length (± 2 nm) were deliberately varied. The deliberate changes in the method do not affect the resolution and tailing factor of analytes significantly (data on file).

Stability

The prepared stock and working solutions were stable for 15 days when stored in refrigerator (2-8˚C). No degraded products were observed during studies. The peak purity was 0.999 or more during the validation studies. On exposure to hydrogen peroxide (25%), HCT and LoP produces several minor degradation products having retention time 2.76, 2.97, 3.42, 3.59, 4.02, 4.18, 5.88, 9.58 and 10.32 min. The percent LoP and HCT remained were 60.85 and 96.72% respectively. In case of alkaline stress, the degradation products were observed at 3.19, 3.98 and 7.99 min. On exposure to the acidic stress analytes gave two minor products (6.83 and 10.72 min) (Table 8). On exposure to different stress conditions, the degradation of LoP and HCT in was lesser than API, which might be due to presence of pharmaceutical excipients. One minor oxidative product was noticed during the studies. The analytes were well separated from the degraded compounds. Figure 4 illustrate the chromatograms obtained during the stability studies.

Table 9. Assay of marketed pharmaceutical formulation and API

	Present method*		**Reported method (R)**	
Drug/formulation	% Assay	SD (RSD)	% Assay	SD (RSD)
HCT	99.4	0.1 (0.10)	99.4	0.23 (0.23)
LoP	99.1	0.6 (0.61)	99.4	0.06 (0.06)
HCT API	99.5	0.02 (0.02)	99.6	0.05 (0.05)
LoP API	99.6	0.01 (0.01)	99.6	0.03 (0.03)

* Student's t test indicates no significant difference ($p>0.05$)

Table 10. Comparison between the Analytical methods

S.N.	Analytical Method (Reference)	Drugs	Column	Detection (λ_{max})	Silent features and advantages	Disadvantage
1	a.) Derivative UV spectroscopy and b.) HPTLC (5. Shah *et al.*, 2001)	LoP and HCT -	- -	a) 271.6nm (LoP), 335. nm for HCT -	(a.) Linearity range-30-70 µg/mL (LoP), and 7.5-17.5 µg/mL (HCT); (b.) Linearity range- 400-1200 ng/spot (LoP); 100-300 ng/spot (HCT). Rapid HPTLC method for analysis.	-
2.	a.) HPLC (6. Erk, 2001) b.) Ratio derivative spectrophotometry	LoP and HCT	RP-YMC pack ODS A A-132 C18 (5 µm, 15 cmx6.0 mm) -	a.) 265 nm b.) LoP-218/236nm; HCT-230/261nm	(a.) Linearity range-1-30 µg/mL (LoP), and 2-20 µg/mL (HCT); (b.) Linearity range-10-50 µg/mL (LoP), and 2-30 µg/mL (HCT), Sensitive, reproducible and accurate method,	Stability studies not reported.
3	CE (8. Hillaert et al., 2001)	HCT and ACEI	Fused-silica capillary (52 cm x 75 µm I.D.)	-	Varied	Run time 20 min, with 2 different buffers
4.	HPLC and derivative spectroscopy (10. Ansari et al., 2004)	LoP	a.) Novapack-ODS (5 µm, 4.0 × 150 mm)	a.) 254 nm (PDA) b.) 232.5 nm	(a) Linearity range- 2-50 µg/mL. Simple; (b.) LOD-0.4µg/mL, LOQ-1.5 µg/mL, Linearity range- 2-50 µg/mL. Simple, accurate, precise method comparable to HPLC.	Stability studies are conducted. No degraded product reported
5.	UV-Partial least squares (11. Maggio, et al., 2008)	LoP and HCT	-	220−274nm	(a.) Linearity range- 4 to 22.2 µg/mL (LoP), (b.) Linearity range -1.06 to 5.70 µg/mL (HCT). Sensitive and precise UV-PLS methods, Comparable to HPLC	Stability studies not carried out
6.	HPLC (12. Argekar and Sawant, 2000)	LoP and HCT	Microbonda pak- C18 column (5 µm, 300 mm x 3.9 mm ID)	270 nm	(a.) Linearity range-2-800 µg/mL (LoP); (b.) 0.5-200 µg/mL (HCT) Sensitive, reproducible, accurate stability indicating method,	Long run time The degraded products in acidic and alkaline stress are not reported.
7.	a.) HPLC and b.) HPTLC (13. Panchal et al., 2010)	LoP and Atorvastatin calcium and	Phenomene x Luna C 18 colum n (250 mm x 4.6 mm i.d., 5 µm)	a) 238 nm (PDA), b) 238 nm	a.) Linearity range-0.5-5 µg/mL b.) Linearity range- 50-500 ng/spot Rapid HPTLC method for analysis.	Narrow range of calibration
8	HPLC (14. Baig et al., 2006)	LoP, Ramipril and HCT	Cosmosil C18 column (5 µm, 150 mm × 4.6 mm i.d.)	215 nm	(a.) Linearity range- 35-65 µg/mL (LoP); (b.) 8.75-16.25 µg/mL (HCT). Simple, rapid, and precise	Injection volume 25 µl, Less sensitive methods Buffer and Peak modiferare required, Stability studies not performed.

9.	HPLC (15. Ozkan., 2001)	Losartan and HCT	LC18 column (5 µm, 150 mm × 4. 6 mm i.d.)	232 nm	(a.) LOD-1.02 ng/ml, Linearity range-0.025-10 µg/mL (LoP); (b.) LOD 4.49ng/ml, linearity range 0.050-10 µg/mL (HCT). Simple, rapid, and precise method for determination of drug in formulation and plasma	Stability studies not performed.
10	HPLC (17.Dinç and Özdemir, 2005)	Enalapril and HCT	Waters Symmetry C18 Column (5 µm, 4.6 × 250 mm)	230, 240, 250, 260 and 270 nm	LOD-0.57 µg/mL, LOQ-1.90 µg/mL, Calibration range 15-40 µg/mL (HCT) Varied wavelength are used for the analysis, Multivariate analysis	Narrow calibration range, Stability studies are not carried out
11	HPLC (18.Carlucci et al., 2000)	LoP and HCT	Cosmosil 18 column (5 µm,150 mm × 4.6 m m i.d.)	230 nm	(a.)LOD-0.08 µg/mL, Linearity range-3-7 µg/mL (LoP); (b.) LOD-0.05 µg/mL, Linearity range 0.5-2 µg/mL (HCT) simple and rapid and allows accurate and precise	Stability studies are not carried out
12	HPLC-PDA (19.Hertzog et al., 2002)	LoP and HCT	Symmetry C8 columns (5 µm, 3.9 × 150 mm)	280 nm	Linearity range- 50 to 150 % level for formulation and linearity range- 0.1-1.5% for degradation studies. HPLC method applicable for separation of drug and degraded products. Degraded products were identified.	Gradient elution. Less sensitivity Run time- 30min.
14.	HPLC (20. Shakya *et al.*, 2013)	HCT and ACEI	Hypersil Gold C-18 column (5 µm, 4.0 × 100 m m)	215 nm	(a.)LOD- 0.3447 µg/mL, LOQ-1.0447 µg/mL, Linearity range -5-35 (µg/mL) (HCT) Simple, rapid and isocratic method for separation of HCT and ACEI in formulation. Developed using design of experiment.	Non- stability indicating method.
13.	HPLC (21.Hafez *et al.*, 2014)	LoP, Amlodipine Besylate, Valsartan and Atorvastatin Calcium	Spherical monomeric C18 column (250 mm×4.6 mm, 5 µm)	240 nm	LOD-0.18 µg/mL, LOQ-0.54 µg/mL, Linearity range-10-60 µg/mL (LoP), Simple analytical method for detection of LoP along with other drugs	stability studies not reported
14	HPLC (24. Smajic *et al.*, 2013)	LoP and HCT	Zorbax C8 (150 x 4.6 mm i.d., 5 µm)	254 nm	(a.) LOD- 80 µg/mL, LOQ-240 µg/mL, Linearity range -10-540 (µg/mL) (LoP); (b.) LOD- 20 µg/mL, LOQ-60 µg/mL, Linearity range- 25 to 125 (µg/mL) (HCT) Developed using design of experiment, effect of methanol content, pH value of the mobile phase, and flow rate on separation of analytes studied	Less sensitive methods. Non- stability indicating method.
15	Proposed HPLC method	LoP and	Hypersil Gold C-18	271nm	(a.) LOD-0.138 µg/mL, LOQ-0.418 µg/mL, Linearity ranges- 2 to 48	Degraded products are separated, but not

	HCT	column (5 µm, 4.6 × 150 m m)	µg/mL(LoP); (b) LOD-0.013 µg/mL, LOQ-0.039 µg/mL, Linearity ranges-0.5 to 12 µg/mL(HCT) Isocratic, accurate, precise efficient, and stability indicating method. Capable of separating different hydrolytic and oxidative products of drug which can be estimated separately. Symmetrical peak shape. Developed utilising statistical design of experiments.	quantized.

Table 11. Summary of the regression and validation parameters

Sn	Parameters	HCT	LoP
1.	Linearity range (µg/ml, n=5)	0.5 - 12	2 - 48
2.	Correlation coefficient (r^2 ± SD)	0.99990 ± 0.00004	0.99996 ± 0.00004
3.	Slope (Mean)	39980.3	6912.7
4.	SEM of Slope	23.2	10.7
5.	Intercept (Mean)	446.3	664.5
6.	SEM of intercept	156.8	289.5
7.	Limit of detection, (LOD, µg/ml)	0.013	0.138
8.	Limit of quantization,(LOQ, µg/ml)	0.039	0.418
9.	Accuracy (%)	99.9 - 100.2	99.6 - 100.9
10	Intra-day Precision (%)	0.17 - 1.45	0.32 - 0.97
11	Inter-day Precision (%)	0.25 - 1.78	1.03 - 1.34
12	Assay of API (Mean ± SD) (%)	99.50 ± 0.01	99.60 ± 0.01
	Assay of Tablets (Mean ± SD) (%)	99.40 ± 0.10	99.10 ± 0.60

Assay

The proposed method was applied for the assay of Tablet formulations of HCT and LoP. The assay of HCT and LoP were 99.4 and 99.1% respectively. The percent purity of HCT and LoP were 99.5 and 99.6% respectively, in case of API. There were no significant difference between the assays obtained using the present method and the reported method[24]. Low value of precision indicates that the method can be used precisely for the estimation of drug in formulations (Table 9).

The present results indicate that the validated method is having advantages over the other methods which are summarised in Table 10. This isocratic method is more sensitive than the stability indicating method reported by Hertzog et al. (2002)[12] and other methods[6,10-11,14,17, 20-21,24].The regression and validation parameters are summarised in Table 11 .

CONCLUSION

A new HPLC method for the estimation of hydrochlorothiazide and losartan potassium has been developed using design of experiments using Box-Behnken design and validated as per ICH guideline. The present method is simple, rapid, economical, accurate, precise,

specific, robust and stability indicating. As the method is developed and optimised using DoE and software program, it can be further optimized as required. The short run time enables the rapid separation of analytes for routine analysis of formulations. No interference has been observed from the excipients or degraded product.

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REFERENCES

(1) Sweetman S. Martindale: the complete drug reference, 33rd edition. Pharmaceutical Press, London. 2002.

(2) Wachtell K., Hornestam B., Lehto M. Slotwiner D.J., Gerdts E., Olsen M.H., Aurup P., Dahlof B., Ibsen H., Julius S., Kjelden S.E., Lindholm L.H., Nieminen M.S., Rokkedal J and Devereux R.B. Cardiovascular morbidity and mortality in hypertensive patients with a history of atrial fibrillation: The losartan intervention for end point reduction in hypertension(LIFE) study. J. *Am. Coll. Cardiol*. 2005; 45 (5): 705-711.

(3) Schmieder R. E., Kjeldsen S. E., Julius S., McInnes G. T., Zanchetti A. And Hua T. A. Reduced incidence of new-onset atrial fibrillation with angiotensin II receptor blockade: the VALUE trial. *J. Hypertens*. 2008; 26: 403-4011.

(4) Naidoo D. P., Sareli P., Marin F., Aroca-Martínez G., Maritz F. J., Jardim P.C., Guerrero A.A., Thompson C.A., Bero T., Drazka J., Kosmalova V., Dumortier T. and Smith R.D. Increased efficacy and tolerability with losartan plus hydrochlorothiazide in patients with uncontrolled hypertension and therapy-related symptoms receiving two monotherapies. *Adv. Ther*. 1999; 16: 187-199.

(5) Shah S.A., Rathod I.S., Suhagia B.N., Savale S.S. and Patel J.B. Simultaneous determination of losartan and hydrochlorothiazide in combined dosage forms by first-derivative spectroscopy and high-performance thin-layer chromatography. *J. AOAC Int.* 2001; 84 (6):1715-23.

(6) Patil K., Bhanu R., Bhoir S. I. and Sundaresan M. Isocratic separation and estimation of losartan potassium and hydrochlorothiazide by packed column supercritical fluid chromatography. *Indian Drugs.* 2003; 40(6): 372-374.

(7) Quaglia M.G., Donati E., Carlucci G., Mazzeo P. and Fanali S. Determination of losartan and hydrochlorothiazide in tablets by CE and CEC. *J. Pharm. Biomed. Anal.* 2002; 29(6): 981-987.

(8) Hillaert S., De Grauwe K., and Bossche W.V. Simultaneous determination of hydrochlorothiazide and several inhibitors of angiotensin-converting enzyme by capillary electrophoresis. *J. Chromatogr*. A. 2001; 924: 439-449.

(9) 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 (4): 603-611.

(10) Ansari M., Kazemipour M., Khosravi F. and Baradaran M. A comparative study of first-derivative spectrophotometry and high-performance liquid chromatography applied to the determination of losartan potassium in tablets. Chem. *Pharm. Bull.* 2004; 52 (10): 1166-70.

(11) Maggio R.M., Castellano P.M. and Kaufman T.S. A multivariate approach for the simultaneous determination of losartan potassium and hydrochlorothiazide in a combined pharmaceutical tablet formulation. *Anal Bioanal Chem.* 2008; 391: 2949-55.

(12) Argekar A.P. and Sawant J.G.A Gradient Reversed Phase High Performance Liquid Chromatography Method for Simultaneous Determination of Hydrochlorothiazide (HCT) and Losartan Potassium (LOS) from Tablets. *Anal. Lett.* 2000; 33: 869-880.

(13) Panchal H.J. and Suhagia B.N. Simultaneous analysis of

Atorvastatin calcium and Losartan potassium in tablet dosage form by RP-HPLC and HPTLC. *Acta Chromatogr*. 2010; 22: 173-187.

(14) Baig M.M., Vaidya V.V., Sane R.T., Menon S.N. and Dalvi K. Simultaneous RP-LC for Losartan potassium, Ramipril and Hydrochlorthiazide in pharmaceutical preparation. *Chromatographia*. 2006; 642: 293-296.

(15) Ozkan S.A. Simultaneous determination of losartan potassium and hydrochlorothiazide from tablets and human serum by RP-HPLC. *J. Liq. Chromatogr. Rel. Technol*. 2001; 24: 2337–2346.

(16) Kolocouri F., Dotsikas Y., Apostolou C., Kousoulos C. and Loukas Y.L. Simultaneous determination of losartan, EXP-3174 and hydrochlorothiazide in plasma via fully automated 96-well-format-based solid-phase extraction and liquid chromatography-negative electrospray tandem mass spectrometry. *Anal. Bioanal. Chem*. 2007; 387 (2): 593-601.

(17) Dinç E. and Ozdemir A. Linear regression analysis and its application to multivariate chromatographic calibration for the quantitative analysis of two-component mixtures. *Il Farmaco.* 2005; 60 (6-7): 591-597.

(18) Carlucci G., Palumbo G., Mazzeo P. and Quaglia M.G. Simultaneous determination of losartan and hydrochlorothiazide in tablets by high-performance liquid chromatography. *J. Pharm. Biomed. Anal.* 2000; 23(1):185-189.

(19) Hertzog D.L., McCafferty J.F., Fang X., Tyrrell R.J. and Reed R.A. 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(3):747-60.

(20) Dawud E.R., and Shakya A.K. HPLC-PDA analysis of ACE-inhibitors, hydrochlorothiazide and indapamide utilizing design of experiments. *Arabian J. Chem.* (2014), http://dx.doi.org/10.1016/j.arabjc.2014.10.052

(21) Hafez H.M., Elshanawane A.A., Abdelaziz L.M., Kamal M.M. Quantitative Determination of Amlodipine Besylate, Losartan Potassium, Valsartan and Atorvastatin Calcium by HPLC in their Pharmaceutical Formulations. *Pharm. Anal. Acta.* 2014; 5: 300. doi:10.4172/2153-2435.1000300.

(22) Yang P., Wang Y. J., Li L., Sun J. and He Z. G. Bioequivalence of Losartan Capsule and Tablet in Healthy Volunteers. *J. Chin. Pharm. Sci.* 2006; 104: 436-439.

(23) Koytchev R., Ozalp Y., Erenmemisoglu A., van der Meer M.J. and Alpan R.S. Combination of losartan and hydrochlorothiazide: in vivo bioequivalence. Arzneimittelforschung. 2004; 54(9A):611-7.

(24) Smajic M., Vujic Z., Mulavdic N. and Brboric J. An Improved HPLC Method for Simultaneous Analysis of Losartan Potassium and Hydrochlorothiazide with the Aid of a Chemometric Protocol. *Chromatographia*. 2013; 76: 419-425.

(25) International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH Harmonised Tripartite Guideline. Validation of Analytical Procedures: Text and Methodology Q2(R1), Step 4 (2010).

(26) Stability ICH Testing of New Drug Substances and Products Q1A (R2), International Conference on Harmonization, IFPMA, Geneva, Switzerland, 2003.

(27) Anderson M. J. and Patrick J. W. RSM Simplified: Optimizing processes using response surface methods for design of experiments, CRC Press, Taylor and Francis group, New York. 2004.

(28) The United States Pharmacopoeia (USP), USP 37/NF 32. United States Pharmacopeial Convention, Rochville, Md, USA, 2014.

التقييم الكروماتوغرافي عالي الكفاية الموصول بالحواس الضوئية المتعددة لدوائي لوسرتان بوتاسيوم والهيدروكلوروثيزايد باستخدام برمجية تصميم التجارب

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ملخص

لقد تم تطوير طريقه بسيطة وسريعة ودقيقة وحساسة للفحص الكروماتوغرافي عالي الكفاية لمادتي لوسرتان بوتاسيومه هيدروكلوروثيلزيد في المستحضرات الصيدلانية. لقد تم تطوير هذه الطريقة والتحقق من صلاحيتها باستخدام برمجية تصميم التجارب، وكان أفضل طور متحرك ذلك الذي يتكون من ماء يحتوي على 0.25 مل/ل من ماده ثلاثي ايثيل امين وميثانول واسيتونتريل بنسب 60:38:30 وتم تعديل الآس الهيدروجيني إلى 7و2 ± 1و0.

وقد تحقق الفصل الكروماتوغرافي بوساطة عمود الفصل Hypersil® Gold C18 وعلى درجة حرارة 25س±2 وقد أجري التحليل على طول موجي 271λ و تم التحقق من صحة الطريقة بإتباع تعليمات المؤتمر الدولي، وكان الزمن المستغرق لعملية الفصل 10 دقائق وكانت الطريقة خطية ضمن المدى من 2–48 ميكروغرام/مل، لمادة لورستان البوتاسيوم ومن 5 و0 – 12 ميكروغرام/ مل لمادة هيدكلوروثيازيد، وتشير الدراسات المجراة أن ثباتية الدواء كانت اعلي خلال الأكسدة من غيرها من ظروف الإجهاد الأخرى.

الكلمات الدالة: التقييم الكروماتوغرافي، لوسرتان بوتاسيوم، برمجية تصميم التجارب.

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