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Quality by design approach (qbd) for the simultaneous determination of anti–hypertensive drugs (candesartan, irbesartan and hydrochlorothiazide) by rp–hplc

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Abstract

A new reverse–phase liquid chromatographic technique has been developed for the separation and determination of anti–hypertensive drugs (Candesartan, Irbesartan and Hydrochlorothiazide) using QbD (Quality by Design). The present method was optimised by introducing experimental design approach to identify the chromatographic conditions where the adequate separation quality in minimal analysis duration. The relationship among independent variables and critical quality attributes is given by experimental design methodology. The segregation was consummated on Sunfire C_{18} type column (150x4.6mm; 5µm particle size) as stationary phase; 0.1% acetic acid in water: acetonitrile (33:77% v/v); 0.7351ml/min as flow rate; detection at 225nm. The chromatographic efficiency was investigated for the composite effects of % organic phase and flow and finely optimized by employing central composite design. The method was validated and was found to be accurate, precise and robust. The method was thrivingly exercised with the marketed formulations.

Keywords: hydrochlorothiazide, irbesartan, candesartan, central composite design, rp–hplc, validation

Abbreviations

QbD: quality by design; CCD: central composite design; IRB: irbesartan; CST: candesartan; HTZ: hydrochlorothiazide; CAN: acetonitrile; PDA: photo diode array; %RSD: percentage relative standard deviation; ICH: international conference on harmonization

Introduction

Sartans are used solely or in alliance with other hypertensives during hypertension therapy.[1,2] These were also used in treatment of diabetic nephropathy in patients suffering with hypertension with type 2 diabetes mellitus and also in congestive heart failure. These angiotensin II receptor antagonists are given in combinations with hydrochlorothiazide, a diuretic that is very effective in treating blood pressure.[3] In modern trends the International Conference on Harmonization (ICH) suggests implementation of Quality by design based experiments in the fields of pharmaceutical product development and also in analytical method development. Optimization of HPLC method very tangled procedure as the separation and other performance criteria depend on various independent variable parameters such as Strength of buffer, mobile phase pH, flow rate, detection wavelength, etc.[4] Any significant interaction between those independent variables may lead to the failure of the univariate procedure as the effect of one variable on the response may be in connection with the levels of the other variables involved in the method optimization. Chemometric approach has become a new and better concept for the RP–HPLC method optimization than the traditional approach based on fluky trial and error methodologies as there is reduction in the number of experiments there by lowering reagent consumption. The experimental design methodology explains the concomitance between the sensitivity of the independent variables and chromatographic parameters that critically attributes the method quality.[5]

Irbesartan (IRB) an angiotensin–II receptor antagonist and also used for the curtailment of renal disease progression in patients with type 2 diabetes.[6] It is chemically known as 2– butyl– 3– ({4–.2– (2H– 1, 2, 3, 4– tetrazol– 5– yl) phenyl phenyl} methyl)– 1,3– diazaspiro.[4] non– 1– en– 4– one ($C_{25}H_{28}N_6O$) 428.53 g/mol.

Candesartan (CST) is an angiotensin II type 1 receptor antagonist.[7] It is chemically tetrazole derivative which is chemically known as – 2– ethoxy– 1– ({4–.2– (2H– 1,2,3,4– tetrazol– 5– yl) phenyl phenyl} methyl)– 1H– 1,3– benzodiazole– 7– carboxylic acid ($C_{24}H_{20}N_6O_3$) with molecular weight 440.45 g/mol.

Hydrochlorothiazide (HTZ) is a thiazide diuretic.[8] Chemically it is known as 6– chloro –1 ,1–dioxo –3, 4– dihydro –2H –1, 2, 4 –benzothiadiazine –7 –sulfonamide ($C_7H_8ClN_3O_4S_2$) with a molecular weight of 297.74 g/mol. The chemical structures of HTZ, IRB and CST were shown in Figure 1A–1C respectively.

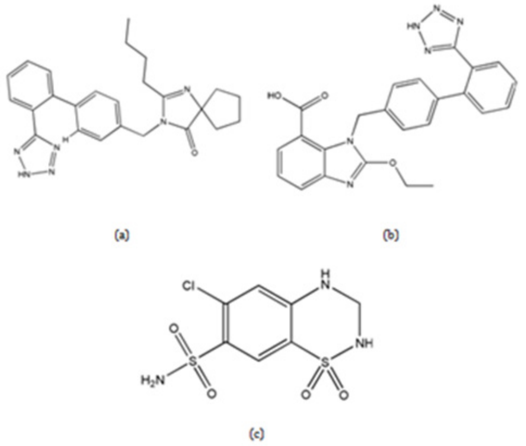

Figure 1 Chemical structures of (a) HTZ, (b) IRB (c) CST.

Literature survey acknowledge that various methods such as LC–MS.[9,10] UPLC.[11] HPLC.[12–17] Micro emulsion LC.[18] HPTLC.[19] spectrofluorometric.[20] and spectrophotometric methods.[21–26] were been developed for simultaneous determination of Irbesartan and Hydrochlorothiazide in biological fluids as well as pharmaceutical formulations.

Similarly, for the simultaneous determination of Candesartan and Hydrochlorothiazide in tablet dosage forms as well as in human plasma few analytical methods such as HPTLC.[27] LC–MS/MS.[28] UPLC–MS/MS.[29] HPLC.[30–40] and spectrophotometric.[41–44] were developed.

Up to our knowledge till date no method was available for simultaneous determination of these three drugs. Therefore, the main aim of the authors is to develop a chemometric–assisted RP–HPLC method possessing statistically optimized chromatographic parameters with simplest mobile phase and is to validate it as per ICH guidelines.[45]

Materials and methods

Instrumentation

CBM–20A/20 Alite model HPLC system of Shimadzu make, equipped with SPD M20A prominence photodiode array (PDA) detector connected to the system Dell Optiplex 790 loaded with LC Solutions v2.0 is rigged for the integrating and processing of chromatograms.

Materials and reagents

Reference standards of CST, IRB and HTZ (purity >99%) was obtained from Sun Pharmaceutical Industries Ltd., India as gift samples. Acetonitrile, glacial Acetic acid, sodium hydroxide (NaOH), Hydrogen peroxide (H_2O_2) and hydrochloric acid (HCl) were acquired from Merck (India). All chemicals used were of analytical grade and used as received. The available marketed formulations are IROVEL–H® (Sun Pharmaceutical Industries Ltd., India) with a label claim: 150mg/12.5mg of IRB/HTZ, and CANDESAR–H® (Ranbaxy Laboratories Ltd., India) with a label claim of 16 mg/12.5mg of CST/HTZ.

0.1% Acetic acid solution (aqueous phase) was made by meticulously transferring 1ml of glacial acetic acid into a 1000ml volumetric flask and make up the volume with HPLC grade water. The climactic solution was sonicated for half an hour and filtered. The stock solutions were prepared by accurately transferring 25mg each of HTZ, IRB and CST in to different 25ml volumetric flasks and all the samples were dissolve using acetonitrile (1000µg/ml) and obligatory supplementary dilutions were fixed from the stock solutions.

Chromatographic conditions

A reverse phase Sunfire C_{18} type column (150mm length × 4.6mm i.d., 5µm particle size) was used as analytical column for the separation. The analysis was fetched by: Waters make Sunfire C_{18} type column (150mm×4.6mm i.d., 5µm particle size); 0.1% acetic acid in water and acetonitrile (33:77, v/v) as mobile phase (Isocratic mode); flow of 0.7531ml/min; UV detection at 225nm. All chromatographic conditions were performed at ambient room temperature (25°C±2°C).

Calculations and software

Chromatographic responses were taken by using LC–Solutions v1.25 which is a data acquiring software by Shimadzu. Design Expert 9.0.3 trial version (Stat–Ease Inc., Minneapolis, MN, USA) has been involved for the experimental design and the selection of the runs. The effects of parameters and their statistical interpretation employed in analytical method development were studied and calculated.

Method validation

Linearity: For the linearity studies a consecution of solutions (0.1–200µg/ml) were projected for each drug from their respective stock solutions and 20µl of each solution was injected in to the HPLC system and the respective chromatograms were chronicled. A calibration curve was charted by considering the concentration of the drug solutions on the x–axis and the cognate peak area on the y–axis and the resulting linear regression equation so attained was adapted for the assay evaluation of marketed formulations.

Precision: The method precision was valuated in terms of repeatability. The precision studies were performed by analysing the samples of HTZ, IRB and CST at three distinctive concentration levels i.e., 5, 10 and 20µg/ml for HTZ and 10, 20 and 50µg/ml for IRB and CST. The % RSD of the three assay values (n=3) was calculated. For inter–day precision the study was conducted on three distinctive days i.e. day 1, day 2 and day 3 while the intra–day precision study held on the same day at distinctive intervals of time.

Accuracy: The accuracy of the developed method was appraised in triplet, spiked at three pre–defined concentration levels (80, 100 and 120%) for all three drugs and their percentage recoveries were calculated. The study was checked out in triplet at 9, 10 and 11µg/ml for HTZ and 18, 20 and 22µg/ml for IRB and CST. The percentage recoveries of all drugs were calculated in each case.

Robustness: The robustness for the developed method was performed by varying the chromatographic circumstances which compraise: flow rate (±0.1ml/min), percentage of organic phase in the mobile phase (±2% acetonitrile, v/v) and wavelength (±2nm). Robustness of the method was reviewed in triplet at a concentration level of 10µg/ml of each drug.

Limit of quantification and limit of detection: The limit of quantification (LOQ) and limit of detection (LOD) were based on the standard deviation of the response and the slope of the charted calibration curve (n=3), as described in ICH guidelines Q2 (R1) (45). LOD and LOQ majorly attributes to the sensitivity of the method.

Assay of marketed formulations: Twenty tablets of each brand of IROVEL – H® (label claim: 150mg/12.5mg of IRB/HTZ), and CANDESAR – H® (label claim of 16mg/12.5mg of CST/ HTZ) were procured from the local pharmacy store, weighed and crushed into fine powder. Powder equivalent to 25mg of IRB and CST respectively of each formulation was accurately weighed and transferred into separate 25ml volumetric flasks and made up to volume with mobile phase. The contents of the volumetric flasks were well sonicated about 30min for the absolute dissolution of the entire drugs. The solutions were centrifuged for 15mins in ultra–centrifuge and the supernatant solution was collected and then filtered through 0.45µm membrane which is used as the stock for the formulations. The prerequisite dilutions were further conducted and analysed. The peak areas were recorded from the respective chromatograms.

Results and discussion

Optimization of experimental conditions

The main aim of developing the RP–HPLC method is to simultaneously determine CST, IRB and HTZ in bulk and tablet dosage form which are separated from each other with good resolution (RS > 2.0), peak shape.tailing factor (TF≤2) and shorter

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analysis time (<10 min) which can be achieved by modifying critical HPLC parameters. During the initiatory experimentation, several mobile phase compositions (% organic phase) as well as flow rates were screened to study their influence on the responses. In the optimization phase, several consequential HPLC parameters whose permutation shows an impact on the separation of the three drugs were identified and to determine the optimum combination and the response pattern a face centred central composite design (CCD) with the two independent variables (% organic phase and flow rate) each at three levels were used as shown in Table 1. A CCD–aided response surface methodology (RSM) based design of experiment was inked to acquire ideal combined effect of % organic phase (acetonitrile) and flow rate on the chromatographic responses. The pooled influence of independent variables each at triplet levels on the chromatographic responses were investigated.

From CCD–aided RSM, nine experimental runs were implemented and the influence of the above said variables at 3 level on the chromatographic responses (resolutions $\left[RS_{(HTZ-IRB)}; RS_{(IRB-CST)}\right]$ tailing factors $\left[T_{HTZ}, T_{IRB} \text{ and } T_{CST}\right]$ and total analysis time.retention time of CST) was investigated (Table 2). The resulted chromatograms from the experiment are depicted in Figure 2a–2i.

Table 1 Experimental variables and coded levels considered in the Central composite design.

Variables	**Levels** **–1 (Low)**	**0 (Medium)**	**1 (High)**
Independent			
% Organic phase (% ACN)	77	82	87
Flow rate (ml/min)	0.6	0.7	0.8
Dependent			
Rs(HTZ–IRB)	= Resolution of IRB		
Rs(IRB–CST)	= Resolution of CST		
THTZ	= Tailing factor of HTZ		
TIRB	= Tailing factor of IRB		
TCST	= Tailing factor of CST		
Totalanalysistime(min)Total analysis time (min)	= Total run time		

Table 2 Experimental runs given by CCD for the two variables at triplet levels and their observed values.

Run	**Independent Variables** **% Organic phase (%ACN)**	**Flow rate (ml/min)**	**Chromatographic Responses** **Rs(HTZ–IRB)**	**Rs(IRB–CST)**	**THTZ**	**TIRB**	**TCST**	**Totalanalysistime(min) Total analysis time (min)**
1	82.0	0.7	9.027	11.944	1.445	1.567	1.384	6.975
2	74.9	0.7	10.326	17.989	1.385	1.404	1.230	10.077
3	82.0	0.5585	9.582	12.238	1.454	1.697	1.498	9.267
4	82.0	0.8414	8.590	11.104	1.470	1.660	1.474	5.686
5	77.0	0.8	9.568	16.037	1.378	1.465	1.324	7.858
6	77.0	0.6	10.273	16.647	1.383	1.562	1.414	10.439
7	87.0	0.6	7.305	8.402	1.493	1.563	1.457	6.158
8	87.0	0.8	7.162	7.917	1.549	1.506	1.309	4.761
9	89.1	0.7	6.340	6.937	1.634	1.560	1.224	4.957

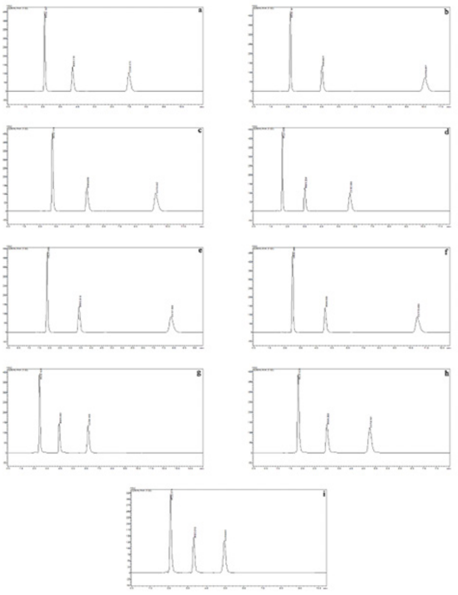

Figure 2 CCD generated trial runs and their respective chromatograms.

Design Expert 9.0 software was employed for RSM computations to produce polynomial models. In the process of analysing the selected model, they were initially evaluated for the fit summary, which gives associability between variables and the responses. The statistical parameters from the analysis of variance (ANOVA) results for this method were listed in Table 3. The values for the model were taken with no transformation. The model terms with probability (Prob > F) (P value) < 0.05 were all highly significant. The high values of the adjusted R^2 for the model reveals that there is a close relation between the experimental and the predicted values of the responses there by indicating the significance and the predictableness of the model. Linear factor equation The independent variable effects and their interaction influences were studied from the computer generated polynomial regression equations (Eq. (1–6)) and given as:

F – Fisher ratio; P – Probability.

$$Rs_{(HTZ-IRB)} = 9.03 - 1.38A - 0.28B + 0.14AB - 0.38A^2 - 3.644E - 003B^2 \quad (1)$$

$$Rs_{(IRB-CST)} = 11.94 - 4.00\,\mathrm{A} - 0.34B + 0.031AB + 0.31A^2 - 0.091B^2 \quad (2)$$

$$T_{HTZ} = 1.46 + 0.079A + \left(9.203E\text{-}003\right)B \quad (3)$$

$$T_{IRB} = 1.57 + 0.033A - 0.026B + (1.000E - 002)AB - 0.057A^2 + 0.042B^2 \quad (4)$$

$$T_{CST} = 1.38 + (2.439E - 003)A - 0.034B - 0.014AB - 0.074A^2 + 0.20B^2 \quad (5)$$

$$Total\ Analysis\,time(\mathrm{min}) = 6.97 - 1.83A - 1.13A - 1.13B + 0.30AB + 0.22A^2 + 0.20B^2 \quad (6)$$

Table 3 Analysis of variance for the screened chromatographic responses.

Variables	**Rs(HTZ–IRB)**		**Rs(IRB–CST)**		**THTZ**		**TIRB**		**TCST**		**Totalanalysistime(min)Total analysis time (min)**	
	F	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
Model	287.08	< 0.0001	1084.06	< 0.0001	31.61	< 0.0001	6.47	0.0148	17.85	0.0007	236.34	< 0.0001
A–% Organic phase	1288.15	<0.0001	5349.91	<0.0001	62.38	<0.0001	5.20	0.0566	0.054	0.8223	834.19	< 0.0001
B–Flow rate	53.82	0.0002	38.06	0.0005	0.84	0.3800	3.21	0.1164	10.55	0.0141	319.15	< 0.0001
AB	6.70	0.0360	0.16	0.6982	–	–	0.24	0.6384	0.96	0.3598	10.94	0.0130
A2	85.45	<0.0001	27.13	0.0012	–	–	13.42	0.0080	43.05	0.0003	10.78	0.0134
B2	0.00785	0.9319	2.39	0.1664	–	–	7.29	0.0306	24.79	0.0016	8.91	0.0204
Adj R2*	0.9917		0.9978		0.8361		0.6950		0.8753		0.9899	
Adeq. Precision**	55.926		107.664		16.441		10.099		15.443		48.656	

Where, A is % organic phase (%ACN) and B is flow rate (ml/min).

The positive value in the equation indicates the favourable response and negative value indicates the inverse effect among the variable and the response respectively. From the equations it is clear that the % organic phase (A) has positive effect on resolution of IRB, resolution of CST and total run time. Whereas, flow rate shows positive effect on resolution of HTZ. On the other hand, the other responses show the mixed type of response.

The perturbation plots (Figure 3a–3f) as well as the three–dimensional (3D) response surface plots (Figure 4a–4f) are very useful for studying the interactions effects of the variables on the responses. The relation between the response variables (i.e., $Rs_{(HTZ-IRB)}$; $Rs_{(IRB-CST)}$; T_{IRB} and T_{CST}; $Total\ Analysis\ time$ and the independent variables is quadratic while in case of T_{HTZ} it is linear.

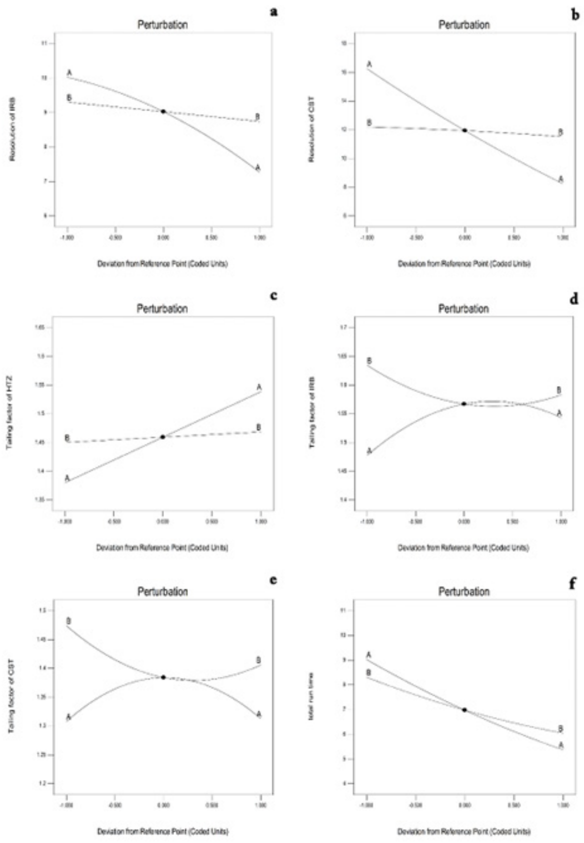

Figure 3 Perturbation plots representing the effect of % organic phase (A) and flow rate (B) on (a) Rs(HTZ-IRB) (b) Rs(IRB-CST) (c) THTZ (d) TIRB (e) TCST (f) Total analysis time.

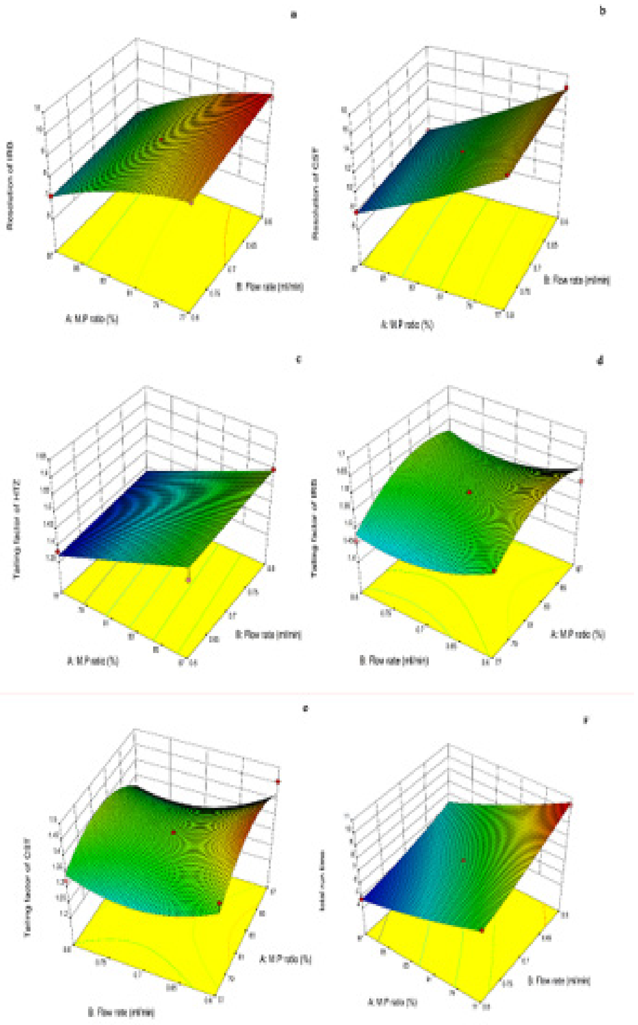

Figure 4 3D Response surface plots representing the effect of % organic phase (A) and flow rate (B) on (a) Rs (HTZ-IRB) (b) Rs (IRB-CST) (c) THTZ (d) TIRB (e) TCST (f) Total analysis time.

The final composition independent variables for the optimization of the HPLC method was achieved by conciliating the various responses (Table 4) to obtain better resolution among the peaks with good tailing factor and minimum analysis time. The bar graph for the desirability in the optimization was shown in Figure 5. Whereas, the desirability ramp for this method was shown in Figure 6 in which the limits for the variables and the desirability criteria were clearly shown. The graphical examination of the desirability was:

Table 4 Desirability criteria for optimized individual responses.

Name	Goal	Lower Limit	Upper Limit
Rs(HTZ–IRB)	maximize	6.34	10.326
Rs(IRB–CST)	maximize	6.937	17.989
THTZ	minimize	1.378	1.634
TIRB	minimize	1.404	1.697
TCST	minimize	1.224	1.498
Totalanalysistime(min) Total analysis time (min)	minimize	4.761	10.439

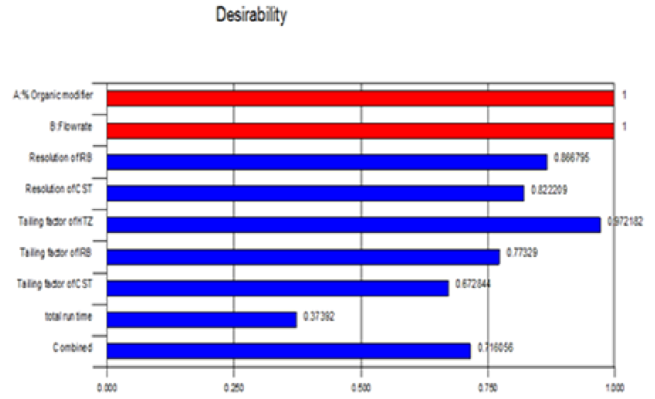

Figure 5 Typical chromatogram of Standard of HTZ, IRB and CST.

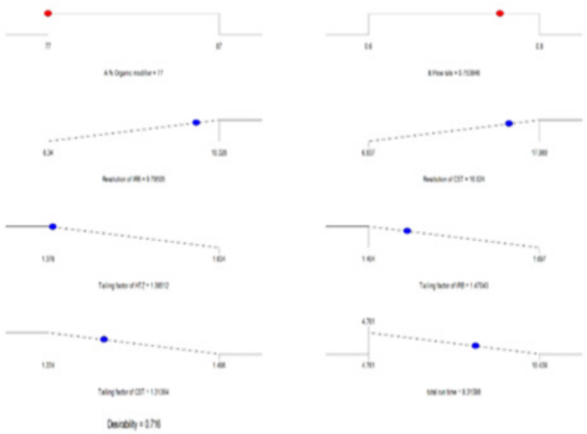

Figure 6 The desirability bar graph for the responses.

$$Percentage\,Predicted\,Error\,(P.E) = \frac{Observed\ -\ Predicted}{Predicted} \text{X}100 \qquad (7)$$

Total 10 runs are given for the design expert software having better desirability which were experimented and the percentage prediction error (P.E) were calculated as per Eq. 7 and shown in Table 5. The run for which the mean percentage prediction error is least compared to the other runs is concluded as the desired method.

Table 5 Table for optimization.

Solution	% Organic Phase (% ACN)	Flow Rate (ml/min)	Responses	Predicted Values	Observed Values	PE	Mean PE
1	77.000	0.7599	Rs(HTZ–IRB)	9.769	9.89	1.237	2.666
			Rs(IRB–CST)	15.995	15.916	–0.496	
			THTZ	1.386	1.369	–1.204	
			TIRB	1.471	1.516	3.050	
			TCST	1.316	1.459	10.839	
			Totalanalysistime(min)Total analysis time (min)	8.243	8.455	2.570	
2	77.000	0.7607	Rs(HTZ–IRB)	9.766	9.829	0.645	2.162
			Rs(IRB–CST)	15.992	15.896	–0.600	
			THTZ	1.386	1.370	–1.136	
			TIRB	1.471	1.492	1.411	
			TCST	1.317	1.455	10.506	
			Totalanalysistime(min)Total analysis time (min)	8.234	8.411	2.144	
3	77.000	0.7587	Rs(HTZ–IRB)	9.775	9.926	1.549	2.673
			Rs(IRB–CST)	16.001	16.040	0.241	
			THTZ	1.386	1.376	–0.690	
			TIRB	1.471	1.508	2.518	
			TCST	1.316	1.440	9.445	
			Totalanalysistime(min)Total analysis time (min)	8.258	8.504	2.976	
4	77.000	0.7571	Rs(HTZ–IRB)	9.781	10.018	2.422	2.171
			Rs(IRB–CST)	16.009	16.128	0.745	
			THTZ	1.385	1.382	–0.247	
			TIRB	1.471	1.467	–0.256	
			TCST	1.315	1.408	7.070	
			Totalanalysistime(min)Total analysis time (min)	8.277	8.549	3.292	

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Table 5 Continued....

Solution	% Organic Phase (% ACN)	Flow Rate (ml/min)	Responses	Predicted Values	Observed Values	PE	Mean PE
5	77.000	0.7564	Rs(HTZ–IRB)	9.784	10.076	2.982	1.813
			Rs(IRB–CST)	16.012	16.009	–0.020	
			THTZ	1.385	1.376	–0.675	
			TIRB	1.471	1.446	–1.678	
			TCST	1.315	1.405	6.868	
			Totalanalysistime(min)Total analysis time (min)	8.285	8.567	3.399	
6	77.000	0.7642	Rs(HTZ–IRB)	9.751	9.75	–0.010	1.402
			Rs(IRB–CST)	15.975	15.962	–0.081	
			THTZ	1.386	1.366	–1.448	
			TIRB	1.472	1.457	–1.006	
			TCST	1.318	1.437	8.992	
			Totalanalysistime(min)Total analysis time (min)	8.193	8.354	1.964	
7	77.000	0.7548	Rs(HTZ–IRB)	9.791	10.216	4.341	1.123
			Rs(IRB–CST)	16.020	16.060	0.252	
			THTZ	1.385	1.368	–1.242	
			TIRB	1.471	1.391	–5.407	
			TCST	1.314	1.379	4.944	
			Totalanalysistime(min)Total analysis time (min)	8.304	8.624	3.848	
8*	77.000	0.7531	Rs(HTZ–IRB)	9.798	10.332	5.447	–0.074
			Rs(IRB–CST)	16.028	16.065	0.233	
			THTZ	1.385	1.365	–1.448	
			TIRB	1.470	1.326	–9.818	
			TCST	1.313	1.329	1.193	
			Totalanalysistime(min)Total analysis time (min)	8.325	8.654	3.950	
9	77.000	0.7735	Rs(HTZ–IRB)	9.711	9.45	–2.688	0.751
			Rs(IRB–CST)	15.929	15.844	–0.532	
			THTZ	1.387	1.366	–1.509	
			TIRB	1.474	1.466	–0.532	
			TCST	1.324	1.429	7.945	
			Totalanalysistime(min)Total analysis time (min)	8.086	8.233	1.821	
10	77.000	0.7149	Rs(HTZ–IRB)	9.960	10.839	8.822	0.569
			Rs(IRB–CST)	16.192	16.149	–0.265	
			THTZ	1.382	1.369	–0.907	
			TIRB	1.473	1.405	–4.630	
			TCST	1.306	1.275	–2.394	
			Totalanalysistime(min)Total analysis time (min)	8.817	9.063	2.786	

From this a combination of 0.1% acetic acid and acetonitrile (33:77, v/v) as mobile phase (Isocratic mode) with a flow of 0.7531 ml/min achieves the desirable responses. At this condition, $RS_{(HTZ-IRB)}$ of 10.332, $RS_{(IRB-CST)}$ of 16.065, T_{HTZ} of 1.365, T_{IRB} of 1.326, T_{CST} of 1.329, and total analysis time of 8.654 were observed and the chromatograph was shown in Figure 7.

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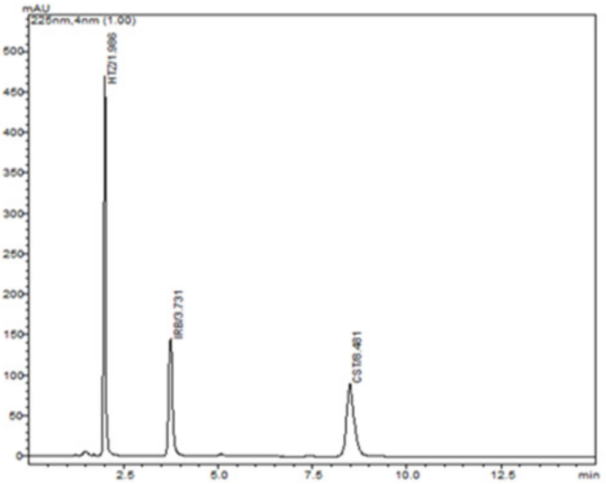

Figure 7 The desirability ramp representing the optimization of the independent variables for the better responses.

Method validation

The method was validated for system suitability, linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), selectivity and robustness (ICH guidelines, 2005).

Linearity

HTZ, IRB and CST follows linearity over a concentration range of 0.01–60µg/ml, 0.05 –120µg/ml and 0.05–100µg/ml respectively (Table 6) with % RSD 0.11–0.45, 0.08–0.42 and 0.10–0.65 for HTZ, IRB and CST respectively. The chromatographic responses of individual samples were shown in Figure 8C–8E respectively for HTZ, IRB and CST respectively. The linear regression equations were found to be $y = 198986x - 11128$ ($r^2 = 0.9999$), $y = 100726x - 5887.5$ ($r^2 = 0.9997$) $y = 117644x + 27207$ ($r^2 = 0.9999$) for HTZ, IRB and CST respectively. The limit of detection (LOD) and limit of quantitation (LOQ) of each drugs were shown in Table 6.

Table 6 Linear regression data for the calibration curve.

Statistical Parameters	Data*		
	HTZ	IRB	CST
Linearity range (µg/ml)	0.01–60	0.05 –120	0.05 – 100
Correlation coefficient (R2)	0.9999	0.9997	0.9999
Slope of curve	198986	100726	117644
Intercept of curve	11128	5887.5	27207

*Mean of three replicates.

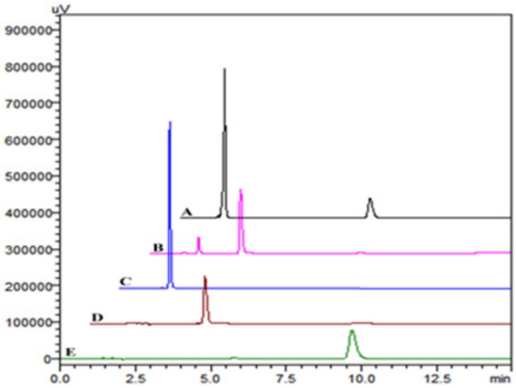

Figure 8 Typical chromatograms [A] CANDESAR-H®, [B] IROVEL–H®, [C]HTZ (10µg/ml), [D] IRB (10µg/ml) and[E] CST (10µg/ml) .

Precision

The intra–day precision of the method was established by assaying the samples of HTZ, IRB and CST at three different concentration levels i.e., 5,10 and 20µg/ml for HTZ and 10, 20 and 50µg/ml for IRB and CST on the same day. The inter–day precision was calculated by assaying the samples of HTZ, IRB and CST at three different concentration levels i.e., 5,10 and 20µg/ml for HTZ and 10,20 and 50µg/ml for IRB and CST on three different days. The % RSD for intra–day precision was found to be 0.06–0.61, 0.34–0.54 and 0.02–0.08 respectively for HTZ, IRB and CST. Whereas the% RSD for inter–day precision was found to be 0.65–1.32, 0.61–1.25 and 0.24–1.19 for HTZ, IRB and CST respectively (Table 7).

Table 7 Precision and accuracy studies of HTZ, IRB and CST.

Analyte	Conc. (µg mL^{-1})	Intra–Day Precision			Inter–Day Precision		
		*Measured conc. (µg mL^{-1}) ± SD	%RSD	SEM	*Measured conc. (µg mL^{-1}) ± SD	%RSD	SEM
	5	5.02±0.03	0.61	0.0177	4.93±0.06	1.32	0.0375
HTZ	10	10.00±0.01	0.08	0.0046	9.89±0.11	1.10	0.0629
	20	20.00±0.01	0.06	0.0065	19.90±0.13	0.65	0.0751
	10	9.82±0.04	0.45	0.0257	9.87±0.12	1.25	0.0715
IRB	20	19.54±0.10	0.54	0.0606	19.86±0.13	0.66	0.0752
	50	49.73±0.17	0.34	0.0981	49.67±0.30	0.61	0.1760
	10	10.03±0.01	0.08	0.0046	9.88±0.12	1.19	0.0678
CST	20	20.02±0.01	0.02	0.0025	19.83±0.15	0.77	0.0879
	50	50.01±0.02	0.05	0.0138	49.86±0.12	0.24	0.0688

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Table 7 Continued...

Accuracy Studies

Analyte	Spiked Conc. (µg mL⁻¹)	Total Theoretical conc. (µg mL⁻¹)	*Conc. Found (µg mL⁻¹) ± SD	%RSD	SEM	%Recovery
	4 (80 %)	9	8.85±0.0318	0.36	0.2042	98.31
HTZ	5 (100 %)	10	9.91±0.0810	0.82	0.4677	99.12
	6 (120 %)	11	10.81±0.0889	0.82	0.4668	98.23
	8 (80 %)	18	17.80±0.0602	0.34	0.1929	98.88
IRB	10 (100 %)	20	19.96±0.0496	0.25	0.1433	99.79
	12 (120 %)	22	21.85±0.0305	0.14	0.0802	99.34
	8 (80 %)	18	17.78±0.0422	0.24	0.1353	98.78
CST	10 (100 %)	20	19.80±0.0644	0.32	0.1858	99.02
	12 (120 %)	22	21.74±0.0193	0.09	0.0507	98.83

*Mean of triplet replicates.

Accuracy

The method accuracy was proved by the recovery test at three different concentrations (80, 100 and 120 %) for all the three drugs. A known amount of standards (10µg/ml) were added to sample solutions and then further diluted to achieve the total concentrations of 18, 20 and 22µg/ml for all the three drugs as described in Table 7. The % recovery for HTZ, IRB and CST was found to be 98.23–99.12, 98.88–99.79 and 98.78–99.02 respectively with %RSD with in acceptance criteria (<2.0 %).

Robustness

The robustness of an analytical procedure is referred as the method's ability to retain unaffected even by small variations in parameters from the original conditions and there by proves the ability of its reliability for routine analysis. The detection wavelength was set at 223 and 227nm (±2nm), the ratio of percentage of 0.1% acetic acid: acetonitrile in the mobile phase was applied as 25:75 and 21:79 (±2, v/v), the flow rate was set at 0.6531 and 0.8531 ml/min (±0.1ml/min). The results obtained for the robustness study were shown in Table 8. From the results it was shown that the retention times and the assays for the test solutions were not much affected by varying the conditions and were in consonance with the results for original conditions. The % RSD value of assay of the sample under original conditions and robustness conditions was less than 2.0% (i.e., 0.018–0.322 for R_t and for assay it is 0.0292–0.2152) indicating that the method is robust.

Table 8 Robustness Studies of HTZ, IRB and CST.

Parameter (Condition)	Analyte	*%Assay ± SD	%RSD	SEM	*Retention Time ± SD	%RSD	SEM
Mobile Phase Flow Rate (± 0.1 mL.min⁻¹)							
	HTZ	98.96±0.0557	0.0563	0.0322	2.019±0.007	0.322	0.0038
(0.6531 mL.min⁻¹)	IRB	98.36±0.1810	0.1840	0.1045	3.844±0.003	0.084	0.0019
	CST	98.48±0.0351	0.0356	0.0202	8.594±0.003	0.029	0.0015
	HTZ	99.34±0.111	0.1120	0.0642	1.922±0.005	0.235	0.0026
(0.8531 mL.min⁻¹)	IRB	101.19±0.1006	0.0994	0.0581	3.672±0.002	0.042	0.0009
	CST	100.20±0.063	0.0627	0.0363	8.354±0.004	0.042	0.0020
Detection Wavelength (±2nm)							
	HTZ	100.34±0.029	0.0292	0.0169	1.985±0.003	0.154	0.0018
(223 nm)	IRB	101.94±0.0436	0.0427	0.0251	3.733±0.002	0.056	0.0012
	CST	100.80±0.215	0.2128	0.1239	8.483±0.002	0.025	0.0012
	HTZ	98.53±0.076	0.0774	0.0440	1.985±0.003	0.127	0.0015
(227 nm)	IRB	98.48±0.0546	0.0555	0.0315	3.731±0.003	0.086	0.0019
	CST	99.32±0.068	0.0686	0.0393	8.483±0.002	0.025	0.0012
Mobile Phase Composition (±2 % Acetonitrile, v/v)							
	HTZ	99.98±0.140	0.1397	0.0807	1.995±0.002	0.077	0.0009
(25:75, v/v)	IRB	100.15±0.1444	0.1441	0.0833	3.823±0.002	0.040	0.0009
	CST	9953±0.120	0.1210	0.0695	8.484±0.002	0.018	0.0009
	HTZ	99.90±0.191	0.1909	0.1101	1.973±0.004	0.183	0.0021
(21:79, v/v)	IRB	99.45±0.2140	0.2152	0.1236	3.682±0.002	0.041	0.0009
	CST	101.06±0.103	0.1022	0.0596	8.472±0.002	0.018	0.0009

*Mean of triplet replicates.

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Analysis of commercial formulations

The proposed method was applied to the available marketed formulations i.e., IROVEL–H® (label claim: 150mg/12.5mg of IRB/HTZ), and CANDESAR–H® (label claim: 16mg/12.5mg of CST/HTZ). The % recovery was found to be 97.69–98.37% for HTZ, 98.47% for IRB and 98.51% for CST (Table 9). The resultant chromatograms obtained for marketed formulations were shown in Figure 8.

Conclusion

A simple isocratic RP–HPLC method for simultaneous determination of HTZ, IRB and CST in bulk and markedly available pharmaceutical dosage form was developed. For this central composite design which is a response surface methodology was adapted to spot out the significant impact of the independent variables such as % organic phase and the flow rate each at triplet levels on the chromatographic responses. The chromatographic responses such at the resolution, theoretical plates, tailing factor and total analysis time were simultaneously optimized with the backing of design of experiments methodology. This multivariate chemometric assisted experimental method development and its validation emphasizes that systematic approach for quality leads to creation of highly budgetary and conscientious chromatographic methods.

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Conflicts of interest

None.

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