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Quality by Design Approach in the Development of a Stability-Indicating RP-HPLC Technique for Levodopa and Benserazide

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Abstract

The study demonstrates development & validation of stability-indicating RP-HPLC technique for simultaneous estimation of Levodopa & Benserazide within a Quality by Design (QbD) framework. Critical method parameters (CMPs) - composition of movable phase, rate of flow, & temperature of column were systematically optimized to achieve predefined critical quality attributes (CQAs), including resolution, tailing factor, and theoretical plates. Separation of compounds in chromatography was done on HPLC system of Waters Alliance make equipped with quaternary gradient pump & detection achieved with UV at 228 nm, with C18 column. The optimized mobile phase consisted of acetonitrile & 0.1% triethylamine buffer (pH 2.5, attuned with orthophosphoric acid) in 46.5:53.5 v/v ratio, delivered at rate of flow at 1.0 mL/min. Retention times for Levodopa & Benserazide were 3.422 & 5.330 minutes, correspondingly. Stress studies under various conditions established, method's stability-indicating capability. Validation performed in accordance with guidelines of ICH Q2(R1) demonstrated excellent linearity (R^2 > 0.999), accuracy (98-102%), precision (RSD < 2%), robustness, & sensitivity. The integration of QbD principles enhanced method robustness, defined a regulatory - compliant design space, and ensured lifecycle reliability. Developed RP-HPLC technique is therefore reliable & versatile tool for routine quality control of Levodopa & Benserazide in formulations.

Keywords: Levodopa, Benserazide, RP-HPLC, Quality by Design, Stability-Indicating Method, Method Validation.

Introduction

The management of parkinson's disease depends heavily on Levodopa as a medicine that produces dopamine (1-3). Medical benefits from Levodopa treatment suffer from rapid degradation in peripheral tissues that decreases availability in brain region (4,5). Benserazide is drug used in management of parkinson's disease (6-8). Benserazide inhibiting the enzyme L-aromatic amino acid decarboxylase in peripheral nervous system (9,10). Benserazide works together with Levodopa (Fig. 1) to enhance its delivery to central areas by decreasing its breakdown in peripheral tissues (11-15).

RP-HPLC represents a standard method for pharmaceutical analysis because it shows excellent analytical capabilities including precision alongside compatibility with complex biological samples (16,17). Stability-indicating method development through RP-HPLC requires special attention because it evaluates pharmaceutical compounds subjected to different stressful

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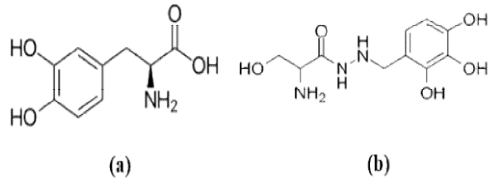

Fig. 1: Chemical structures of (a) Levodopa & (b) Benserazide

conditions in order to protect therapeutic outcomes and defend patient safety (18-23).

The pharmaceutical industry now widely uses QbD principles for developing analytical techniques throughoutpresent decade (24). QbD requires a systematized scientific process which includes risk identification through Design of Experiments (DoE) to identify & control essential method variables (25). A method design space becomes established when this strategy is implemented which enables consistent method performance throughout specific operating conditions (26).

A novel, robust & stability-indicating RP-HPLC system is being established in this study to determine Levodopa and Benserazide jointly in their bulk and tablet formulations (27,28). QbD principles allow method to meet regulatory standards according to ICH guidelines through optimization of important chromatographic parameters (29,30).

Materials and Methods

Chemicals and Reagents

The analysis employed pharmaceutical-grade Levodopa & Benserazide standards obtained from authenticated sources. Acetonitrile & triethylamine (TEA), both of HPLC grade, were used as solvents. Orthophosphoric acid was utilized to adjust pH of aqueous component of mobile phase. Milli-Q purified water was used throughout study. All solvents & solutions were filtered via 0.45 µm membrane filters prior use to ensure particulate-free analysis.

Instrumentation & Analytical Conditions

Chromatographic separation was done using HPLC system of Waters Alliance make equipped with quaternary gradient pump, UV detector, auto sampler & temperature-controlled column oven. A reverse-phase C18 column was used as stationary phase. After a series of optimization trials, mobile phase was finalized as a 46.5:53.7 v/v mixture of acetonitrile & 0.1% TEA buffer, with latter attuned to pH 2.5 using OPA. Temperature of column was tunned on 30°C, & rate of flow was fixed at 1.0 mL/min. Detection was done at 228 nm, & 10 µL of each sample was injected for analysis.

Preparation of Standard & Sample Solutions

To prepare standard, 100 milli gram of Levodopa & 25 milli gram of Benserazide were precisely weighed & moved to volumetric flask of capacity 10 mL. Contents were dissolved in mobile phase with sonication & then diluted to volume. Working standard solution was prepared with further dilution of this stock. For sample solution, twenty tablets were weighed to determine average tablet weight. Quantity equivalent to single tablet was moved to volumetric flask, dissolved in mobile phase with sonication for 30 mins, & filtered using 0.45 µm membrane. Filtrate was appropriately diluted to obtain final test solution.

Quality by Design (QbD) Experimental Design

The method development was done through QbD to ensure robustness & reliability. Based on preliminary risk assessment, three CMPs were identified: composition of mobile phase, flow rate, & temperature of column. Box-Behnken Design (BBD) was employed to study influence of these variables, total of 17 randomized experimental runs were conducted as per BBD matrix. Statistical modelling & response surface analysis were performed using Design-Expert® software. Critical quality attributes (CQAs) such as resolution, tailing

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factor, & theoretical plates were evaluated to construct design space that ensures optimal method performance.

Method Validation

Established method was validated in harmony with ICH Q2(R1) guidelines. Specificity was established by ensuring lack of interfering peaks at analyte retention times in both blank & stressed samples. Linearity was established across suitable concentration range for both analytes, with correlation coefficients exceeding acceptable threshold. Precision was confirmed through intra-day & inter-day replicates, with results articulated as RSD. Accuracy was verified in the course of recovery studies using standard addition technique at multiple concentration levels. Robustness was evaluated by introducing small variations in method parameters, such as flow rate & mobile phase ratio, to assess their effect on performance. LOD & LOQ were measured based on signal-to-noise ratio. Forced degradation studies were conducted under acid, alkali, peroxide, reductive, thermal, photolytic & hydrolysis conditions to evaluate method's stability-indicating capability.

Results

Design of Experiment & Model Fitting

The BBD was employed to study control of composition of mobile phase, flow rate, & temperature of column on three critical quality attributes: resolution, tailing factor, & theoretical plates, total of 17 experimental runs were conducted out (Table 1).

Regression analysis revealed that quadratic model best fit data for all three responses. Model for resolution yielded an R^2 of 0.962, while tailing factor & theoretical plate's models had R^2 values of 0.783 &

Table 1: Experimental design matrix with responses

Std	Run	A: Mobile phase composition	B: Flow rate	C: Column temperature	Resolution	Tailing factor	Theoretical plates
2	1	50.0	0.800	30.0	2.80	1.20	3500
7	2	30.0	1.00	35.0	3.10	1.10	3200
17	3	40.0	1.00	30.0	3.00	1.20	3400
5	4	30.0	1.00	25.0	2.90	1.30	3100
6	5	50.0	1.00	25.0	3.20	1.20	3600
8	6	50.0	1.00	35.0	3.30	1.10	3700
14	7	40.0	1.00	30.0	3.00	1.20	3400
16	8	40.0	1.00	30.0	3.00	1.20	3400
15	9	40.0	1.00	30.0	3.00	1.20	3400
4	10	50.0	1.20	30.0	3.40	1.10	3800
13	11	40.0	1.00	30.0	3.00	1.20	3400
11	12	40.0	0.800	35.0	3.10	1.30	3300
12	13	40.0	1.20	35.0	3.50	1.10	3600
9	14	40.0	0.800	25.0	2.80	1.30	3200
10	15	40.0	1.20	25.0	3.30	1.20	3400
3	16	30.0	1.20	30.0	3.20	1.10	3300
1	17	30.0	0.800	30.0	2.90	1.30	3100

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0.996, respectively. ANOVA confirmed statistical significance of models ($p < 0.05$). Interaction & 3D response surface plots (Fig. 2) illustrated the effects of mobile phase composition & flow rate on resolution, tailing factor, & theoretical plates. Resolution improved with higher acetonitrile up to an optimum, while flow rates above 1.0 mL/min reduced it. Tailing factor was mainly affected by mobile phase, with little change across flow rates. Theoretical plates increased with higher acetonitrile & moderate flow rates. These plots revealed variable interactions & defined robust design space meeting all acceptance criteria (Resolution ≥ 3.0, Tailing ≤ 1.3, Plates ≥ 3000).

Optimization & Design Space

Using desirability function analysis, optimal chromatographic conditions were determined: 46.5% acetonitrile, 1.06 mL/min flow rate, & 29.1°C column temperature. These settings maximized resolution & plate count while maintaining an acceptable tailing factor. Predicted values under optimal circumstances are presented in (Table 2).

System Suitability

System suitability tests under optimized conditions produced robust results. Resolution between Levodopa & Benserazide exceeded 3.0, tailing factors were below 1.3, & number of theoretical plates surpassed 3000 (Table 3).

Table 2: Chromatographic conditions optimized & predicted responses

S. No.	Parameter	Optimized Value / Response
1	Mobile Phase Composition (%)	46.5
2	Flow Rate (mL/min)	1.06
3	Column Temperature (°C)	29.1
4	Resolution	3.23
5	Tailing Factor	1.19
6	Theoretical Plates	3,610

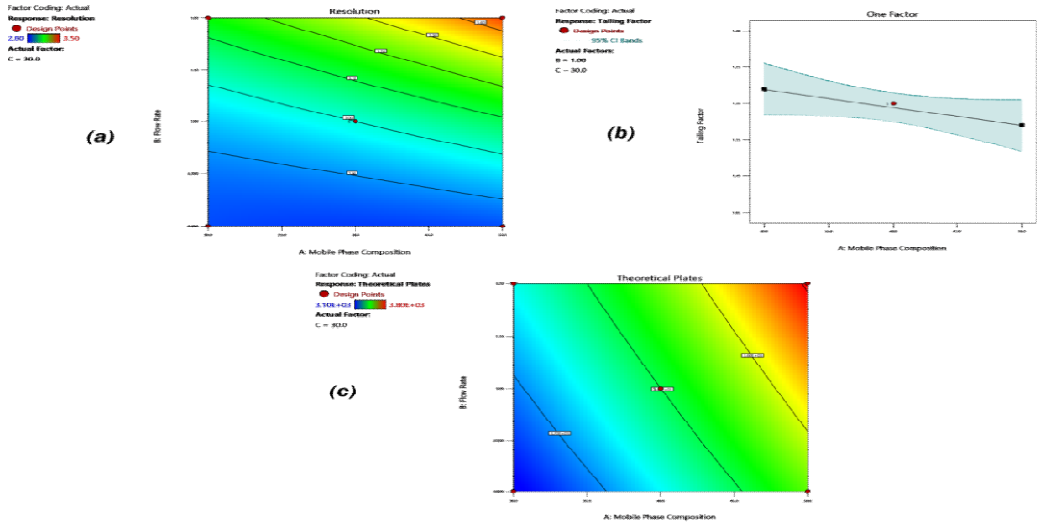

Fig. 2: Interaction & 3D response surface plots illustrating the effects of acetonitrile percentage & flow rate on (a) Resolution, (b) Tailing factor, & (c) Theoretical plates. Shaded regions represent the design space where acceptance criteria (Resolution ≥ 3.0, Tailing ≤ 1.3, Plates ≥ 3000) are satisfied

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Table 3: System suitability parameters			
S. No.	Constraint	Levodopa	Benserazide
1	Retention time (min.)	3.422	5.330
2	Plate count	12844	15279
3	Tailing factor	1.19	1.07
4	Resolution	--	6.69
5	% RSD	0.25	0.14

Linearity

Calibration curves for Levodopa (25-150 micro gram/milli litre) & Benserazide (6.25-37.5 micro gram/milli litre) showed excellent linearity. Correlation coefficients (R^2) were 0.9996 & 0.9999, respectively. Regression equations are presented in (Table 4), & calibration curves are shown in (Fig. 3).

Precision

System precision, repeatability, & inter-day precision data indicated excellent reproducibility. %RSD was below 2% for both analytes across all levels (Table 5).

Table 4: Linearity data for Levodopa & Benserazide				
S. No.	Levodopa		Benserazide	
	Conc. (micro gram/milli litre)	Peak area	Conc. (micro gram/milli litre)	Peak area
1	25	769383	6.25	196213
2	50	1558638	12.50	392784
3	75	2424572	18.75	585647
4	100	3130368	25.00	785351
5	125	3855920	31.25	983646
6	150	4589679	37.50	1155942
Regression equation	y=30734.06x+25597.04		y=31058.62x+3305.54	
Slope	30734.06		31058.62	
Intercept	25597.04		3305.54	
R^2	0.99960		0.99986	

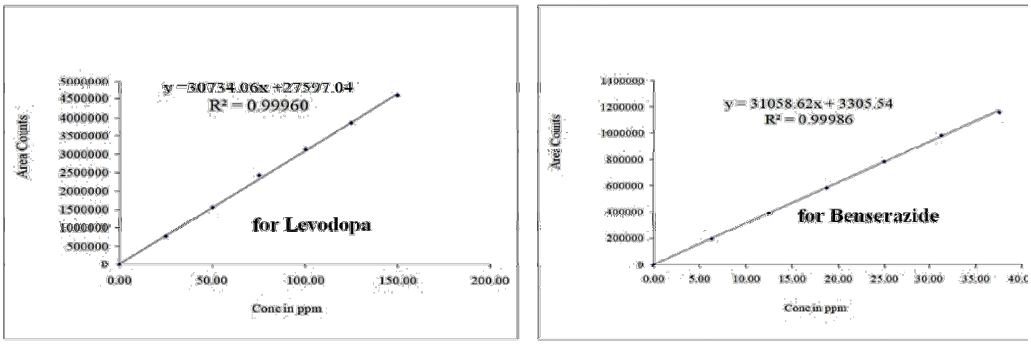

Fig. 3: Calibration curves for Levodopa & Benserazide.

RP-HPLC Technique for Levodopa and Benserazide

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Table 5: Precision data for Levodopa & Benserazide

S. No.	Levodopa		Benserazide	
	Conc. (micro gram/milli litre)	Peak area	Conc. (micro gram/milli litre)	Peak area
1	100	3137401	25	786065
2	100	3139513	25	788651
3	100	3142170	25	787250
4	100	3130128	25	788798
5	100	3126354	25	786512
6	100	3147434	25	787446
Mean	3137167		787454	
SD	7777.770		1104.310	
% RSD	0.25		0.14	

Table 6: Accuracy data for Levodopa & Benserazide

Accuracy results for Levodopa					
%Conc. (at specification level)	Peak area	Amount added (mg)	Amount found (mg)	% Recovery	% Mean recovery
50%	1572882	50.0	50.14	100.3	100.9
100%	3181021	100.0	101.40	101.4	
150%	4759614	150.0	151.72	101.1	
Accuracy results for Benserazide					
50%	393069	12.5	12.48	99.8	99.8
100%	785564	25.0	24.94	99.8	
150%	1178465	37.5	37.41	99.8	

Accuracy

Recovery studies at 50%, 100%, & 150% concentration levels confirmed method's accuracy. Average percentage recovery ranged from 99.8% to 100.9% for both drugs, well within acceptable limits (Table 6).

Robustness

Robustness was understudied by modifying rate of flow (±0.1 mL/min) & organic phase composition (±5%). No significant changes in retention time, resolution, or peak symmetry were observed, confirming method's resilience to small operational changes and the results are within the acceptance criteria (Table 7).

LOD & LOQ

The limits of detection & quantitation were determined to be 0.300 micro gram/milli litre & 1.000 micro gram/milli litre for Levodopa, & 0.075 micro gram/milli litre & 0.250 micro gram/milli litre for Benserazide, correspondingly. These values demonstrate method's high sensitivity.

Forced Degradation Studies

Degradation studies revealed that Levodopa & Benserazide were stable under various stress conditions. Degradation products did not interfere with analyte peaks. Chromatograms (Fig. 4) confirmed clear separation of parent compounds from

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Table 7: Robustness results under varied chromatographic conditions

Parameter	Circumstance	Retention time (min.)	Peak area	Tailing	Plate count	%RSD
	Levodopa					
Flow rate change (mL/min.)	Less flow (0.9 mL)	3.566	3062369	1.22	12920	0.50
	More flow (1.1 mL)	3.247	3331487	1.13	12733	0.98
Organic phase change	Less org. (41.5:58.5% v/v)	3.708	2853640	1.18	12976	0.57
	More org. (51.5:48.5% v/v)	3.133	3454324	1.10	12741	0.48
Benserazide						
Flow rate change (mL/min.)	Less flow (0.9 mL)	5.505	765204	1.10	15350	0.25
	More flow (1.1 mL)	5.153	797462	1.02	15197	0.30
Organic phase change	Less org. (41.5:58.5% v/v)	5.674	759849	1.14	15384	0.64
	More org. (51.5:48.5% v/v)	5.041	803567	1.05	15143	0.40

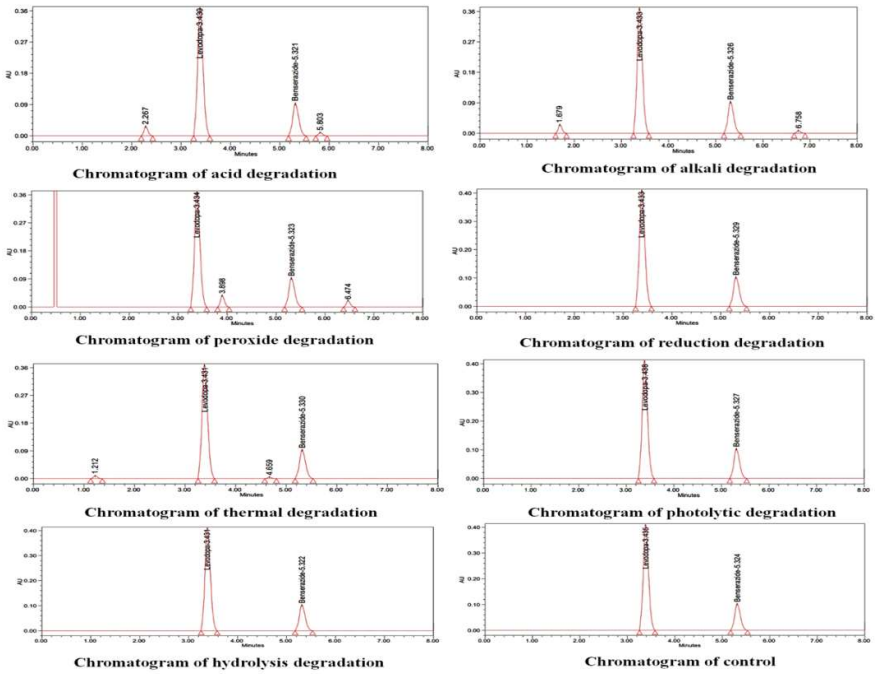

Fig. 4: Chromatograms of degradation samples under stress conditions

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degradation products, validating stability-indicating nature of method.

Discussion

The present study successfully implemented QbD, employing Box-Behnken Design to optimize & validate an RP-HPLC technique for the concurrent evaluation of Levodopa & Benserazide. Unlike previously reported methods, which often relied on one-factor-at-a-time (OFAT) approaches or lacked systematic evaluation of critical method parameters, the QbD framework allowed structured investigation of movable phase composition, rate of flow, & temperature of column on critical quality attributes (CQAs) such as resolution, tailing factor, & theoretical plates.

Regression analysis & ANOVA confirmed that quadratic model adequately described the response surfaces, with high R^2 values (0.962 for resolution, 0.783 for tailing factor, & 0.996 for theoretical plates), demonstrating excellent model predictability & robustness. Interaction & 3D response surface plots highlighted significant synergistic & antagonistic effects, particularly between organic solvent content & flow rate, enabling precise identification of an optimal design space-feature largely absent in conventional method development studies.

Optimization through desirability function analysis predicted ideal conditions at 46.5% acetonitrile, 1.06 mL/min flow rate, & 29.1°C column temperature. Experimental verification confirmed these predictions, with system suitability parameters surpassing the acceptance criteria & demonstrating high resolution (>6.5), minimal tailing, & elevated theoretical plate counts. This predictive reliability exemplifies one of the key advantages of QbD, which contrasts with earlier methods where post-development adjustments were often required to meet acceptance criteria.

Linearity, precision, & accuracy were outstanding, with correlation coefficients exceeding 0.999 & %RSD below 2%, comparable or superior to previously reported methods. Recovery studies ranged from 99.8% to 101.4%, reflecting high method reliability without interference, which addresses limitation noted in some prior HPLC methods where analyte quantification was affected by excipients or degradation products. Robustness evaluation further confirmed that small deliberate variations in flow rate & organic content had negligible impact, underscoring the method's resilience-a hallmark of QbD-guided development. Sensitivity was demonstrated through low LOD & LOQ values, particularly for Benserazide (0.075 & 0.250 µg/mL), ensuring applicability for trace-level detection.

Importantly, stress studies established method's stability-indicating capability, with all degradation peaks well-resolved from the parent compounds under stress conditions. This not only meets regulatory expectations but also provides an advantage over earlier methods where degradation products were often insufficiently characterized or unresolved.

The QbD-based RP-HPLC method developed herein is precise, accurate, robust, & stability-indicating, fully compliant with ICH Q2(R1) guidelines. Compared with traditional approaches, the QbD strategy provides deeper understanding of method performance, ensures consistent quality across variable operational conditions, reduces the risk of method failure, & facilitates regulatory acceptance. This makes it highly suitable for QC of Levodopa & Benserazide, offering systematic, predictive, & reliable alternative to conventional method development practices.

Conclusion

A robust & reliable RP-HPLC technique for concurrent determination of Levodopa & Benserazide in bulk & tablet formulations was successfully developed & validated using QbD-based approach. Implementation of Box-Behnken Design facilitated systematic evaluation of CMP-movable phase composition, rate of flow, &

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temperature of column on key analytical performance attributes, including resolution, tailing factor, & theoretical plates. The optimized method exhibited excellent linearity, precision, accuracy, & robustness, fully complying with ICH Q2(R1) guidelines.

Importantly, the method demonstrated high sensitivity with low limits of detection & quantification & effectively separated analytes from degradation products, confirming its stability-indicating capability. Adoption of QbD principles not only enhanced process understanding & ensured consistent method performance across the defined design space but also aligns with regulatory expectations for systematic, risk-based method development. From an industrial perspective, this approach reduces the likelihood of method failure, streamlines regulatory submissions, & supports efficient quality control & stability testing of Levodopa & Benserazide in pharmaceutical products. Thus, the QbD-guided RP-HPLC method represents strategic advancement for both regulatory compliance & industrial application.

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