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Design, Development, and Validation of a Stability Indicating RP-HPLC Method for Determination of Diazoxide and Organic Impurities in an Oral Suspension Formulation Using AQbD Approach

Kopperundevi[1], Basuvan Babu[2], Rajendran SD[3], Jeya Prakash[4], Karthic.M[5]

[1,2&5] Department of Pharmaceutical Analysis, JSS College of Pharmacy, Ooty, Nilgiris, Tamil Nadu-643001, India.
[3]Scitus Pharma Services Private Limited, Chennai-600 124, Tamilnadu, India.
[4]MB School of Pharmaceutical Sciences, Mohan Babu University, Sree Sainath nagar, Tirupati-517102, India

ABSTRACT: An RP-HPLC method for the determination of diazoxide content and organic impurities from an oral suspension formulation was established and successfully validated. AQbD was applied to develop this stability-indicating assay method. By optimizing three main factors – buffer pH, organic modifier ratio and flow rate – through CCD, a scientifically and statistically valid MODR was determined. Selectivity was improved by choosing biphenyl as the stationary phase since it facilitates the formation of better aromatic interactions between the analytes, resulting in RRs > 2.0 between diazoxide and its impurities. Forced degradation under acidic, basic, oxidative, thermal and photolytic conditions verified the stability-indicating potential of the developed method, with mass balance maintained at 98–102% level. The study also included a detailed validation process in accordance with ICH Q2 (R2) and a lifecycle-based analytical control strategy was formulated, as stipulated by ICH Q14. Results obtained from method validation were within acceptable limits for the MODR with respect to linearity ($r^2 \geq 0.9996$), percent RSD < 2.0%, and 98.7-101.4% accuracy. Overall, the developed assay procedure is ideal for quality control, stability and regulatory purposes for diazoxide oral suspension formulations.

1. INTRODUCTION

The concept of impurity profiling is one of the important aspects of regulatory compliance since it guarantees safety, efficacy, and quality of pharmaceutical products. Organic impurities exceeding the reporting threshold must be identified, characterized and quantified based on guidelines provided by ICH Q3A (R2) and Q3B (R2) [1,2].

The recent regulation updates, such as ICH Q14 and the amended ICH Q2 (R2), highlight the lifecycle concept for analytical procedures, which is based on risk assessment, experimental design and validation processes. Simultaneously, the implementation of Quality Risk Management guidelines according to ICH Q9 (R1) and Good Clinical Practice guidelines according to ICH E6 (R3) additionally stress the need for scientifically sound, reliable and fit-for-purpose analytical control approaches [3,8].

Diazoxide belongs to the benzothiadiazine family, which consists of a ring structure along with a sulfonamide functional group and thus is prone to undergo oxidation and hydrolysis [9,10]. The presence of aromatic heterocycles makes this analysis very important from a chromatographic point of view.

In the current studies, biphenyl phases have been found to give excellent selectivity because of π-π, dipole-dipole interaction and shape interaction. Comparisons have shown that biphenyl columns have better separations when compared with regular C18 columns. [12,15–17].

This paper describes the development and validation of a robust RP-HPLC method which can be used for the analysis of impurities present in diazoxide, marketed as an oral suspension.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

The Diazoxide working standard (purity 99.6%, Lot No. PRS/3040/01), Diazoxide oral suspension (RBMP-019-089b) and Placebo (RBMP-013-033) were obtained from NURAY Chemicals Pvt. Ltd.. HPLC-grade acetonitrile and methanol were purchased from Kesari Scientific Chemicals. Ammonium formate (analytical grade, ≥98.0%) was procured from Sigma-Aldrich. Formic acid (LC-MS grade) was obtained from Moltek Life Sciences. Water was purified using a Milli-Q water purification system (Millipore, resistivity ≥18.2 MΩ·cm).

2.2 Sample Preparation Procedure

Buffer Preparation:

Weighed about 1.56 g of Sodium Dihydrogen Phosphate accurately and dissolved in 1000 mL of purified water. Mix well. The solution was thoroughly mixed and adjusted to pH to 2.50 ± 0.05 by the addition of dilute phosphoric acid as a layer wise. The prepared solution was filtered by using 0.45 µm nylon membrane filter and degassed followed by sonication.

Mobille Phase A:

It was prepared by transferring 850mL of the prepared buffer solution with the addition of HPLC- grade methanol into 1000mL container in the ratio of 85:15(v/v). The mixture was stirred well, filtered through a 0.45µm nylon membrane, and degassed by sonication.

Mobile Phase B:

Mobile Phase B is a critical solvent mixture in HPLC, prepared by combining two high-purity solvents: HPLC-grade acetonitrile and methanol. The preparation involves mixing these solvents in a precise volume ratio of 70:30 (v/v), where 700 mL of acetonitrile is blended with 300 mL of methanol in a 1000 mL container. To ensure the homogeneity and purity of the mobile phase, the mixture is thoroughly mixed, then filtered through a 0.45 µm membrane filter to remove particulate matter. Additionally, the solution is degassed by sonication, which eliminates dissolved gases that could interfere with chromatographic detection and consistency.

Diluent:

The diluent serves as the medium for preparing all subsequent solutions unless otherwise directed. It is formulated by mixing a buffer and methanol in equal volumes, 50:50 (v/v). This balanced mixture provides a stable environment that supports the solubility and stability of analytes and standards throughout the HPLC process.

Standard Stock Solution:

The standard stock solution is prepared with meticulous care to ensure accurate quantification. A precisely weighed 50 mg of Diazoxide Reference Standard (RS) is placed into a 100 mL volumetric flask. About 80 mL of the prepared diluent is added to the flask, and the mixture undergoes sonication until the Diazoxide fully dissolves. The solution is then brought to the final volume with diluent and mixed thoroughly, resulting in a stock concentration of 500 µg/mL. This stock solution serves as the benchmark for calibration and quality control during HPLC analysis.

Intermediate Stock: To 100mL volumetric flask was filled with 5.0mL of aliquot of standard stock solution, diluted well to the volume marked. The concentration of the prepared solution as 25µg/mL.

Standard Working Solution:

A 100 mL volumetric flask was filled with a 4.0 mL aliquot of the intermediate stock solution, which was then diluted to volume using the diluent. The working standard solution's ultimate concentration was roughly 1.0 µg/mL, which is equivalent to the linked compounds' 100% working level.

Impurity Stock Solutions:

Stock solution were individually of each impurity standard (Impurity A,B,C and D) were prepared at the concentration of 1mg/ml. To the combined impurity spiking solution was subsequently prepared at the concentration range of 10µg/ml with relative 10% diazoxide , which was diluted too.

Sample Preparation for Oral suspension:

Weighed about 2000mg of Diazoxide oral suspension with 100mg of diazoxide, which was transferred to 200ml volumetric flask. To this HPLC - grade methanol was added, the flask content was mixed throughly for the specific time of 15 minutes with sonication. Followed by addition of 80mL buffer was added, and the mixture shaken for 30minutes. The solution was made up to the volume by using buffer solution with the final concentration of 500µg/mL. The sample was filtered by using 0.45µm PVDF membrane filter before the spectral analysis.

Control sample:

A total gm of about 2.0289g of the final prepared product was taken and transferred into 200mL volumetric flask. To theis 100mL of HPLC-grade methanol was added, the mixture kept under sonication for 15minuts. Subsequently, buffer solution about 80mL was added, with vigoured shaking. The volume was maked upto the mark of the volumetric flask approximately with the concentration of 500µg/mL. The prepared final solution was passed through the filter paper 0.45 µm PVDF membrance filter before the chromatogram.

2.3 Instrumentation

Chromatographic analysis was performed using an Agilent 1260 Infinity II HPLC system equipped with a quaternary pump (G7111B), high-performance autosampler (G7167B), thermostatted column compartment (G7116A) and a diode array detector (DAD, G7115A). Data acquisition and processing were carried out using Agilent Open LAB CDS Chem Station software (version 2.5, validated). The column used was a Phenomenex Kinetex Biphenyl column (4.6 × 50 mm, 2.6 µm particle size).

2.4 AQbD Strategy and Experimental Design

AQbD strategy was applied to establish important method parameters such as pH of the buffer, percent of organic modifier and flow rate. The Central Composite Design (CCD) approach was used to evaluate the impact of these parameters on critical analytical performance attributes like resolution and tailing factor. The response surface methodology was used to establish the operational space for the method [3,5,6,7].

2.5 Forced Degradation Studies

Forced degradation studies were carried out using acidic, basic, oxidative, thermal and photolysis stress conditions to investigate specificity and forced degradation patterns of the method. The goal was to produce degradants representing samples with partial destruction of the analyte and separation of the active drug substance from all identified impurities [18,19]. Acid Stress: 2.0204 g of finished product + 20.0 mL of 1N HCl, heated in a water bath at 80°C for 24 hours, neutralized with 20.0 mL of 1N NaOH. Processed as control. Alkaline Stress: 2.0079 g of finished product + 20.0 mL of 1N NaOH, heated in a water bath at 80°C for 24 hours, neutralized with 20.0 mL of 1N HCl. Processed as control. Peroxide (Oxidative) Stress: 2.0081 g of finished product + 20.0 mL of 3% H_2O_2, heated in a water bath at 80°C for 24 hours. Processed as control. Thermal stress: 2.0327 g of finished product heated in an oven at 80°C for 24 hours, then dissolved as per control preparation. UV Light (Photolytic) Stress: 1.9978 g of finished product kept in a UV chamber for 24 hours, then dissolved as per control preparation.

2.6 Method Validation

Method validation was carried out in line with ICH Q2 (R2) in terms of specificity, linearity, precision, accuracy, sensitivity, robustness and solution stability. Linearity was determined in the defined range of concentrations, precision by conducting replicate injections, accuracy by recovery experiments, robustness by deliberate changes in pH, flow rate and temperature [4].

3. RESULTS AND DISCUSSION

3.1 AQbD Critical Method Parameters

Based on AQbD risk assessment, the three factors optimized by Central Composite Design (CCD) employed using Design-Expert software V13 were: (X1) Buffer pH — studied range 2.30–2.70; optimum 2.50; (X2) Organic modifier ratio (% Mobile Phase B at 12 min) — studied range 3–7%; optimum 5%; (X3) Flow rate — studied range 0.65–0.95 ml/min; optimum 0.80 ml/min. The critical method attributes monitored were resolution (Rs) and tailing factor (Tf) for the diazoxide peak. The pH variation of ±0.20 and column temperature variation of ±5°C do not significantly affect the diazoxide retention time or system suitability, defining the practical method parameters as table 1.

Table 1. AQbD Parameters

Parameter	Studied Range	Robustness Range Tested	Optimum Value	Method Operable Design Region
Buffer pH (X1)	2.30 – 2.70	2.30, 2.50, 2.70	2.5	2.30 – 2.70
Column Temperature (X2)	25 – 35°C	25°C, 30°C, 35°C	30°C	25 – 35°C
Flow Rate (X3)	0.65 – 0.95 ml/min	0.8 ml/min (nominal)	0.80 ml/min	0.75 – 0.85 ml/min

3.2 Chromatographic Selectivity

Initial experiments involving regular C18 columns showed some degree of overlapping among oxidative degradates. Switching over to a biphenyl stationary phase has significantly improved selectivity, resulting in separation ratios (Rs) greater than 2.0 for all impurity pairs and other chromatographic conditions were shown in table 2. Such selectivity improvement can be attributed to π-π stacking interaction that occurs between the aromatic benzothiadiazine ring structure and the biphenyl surface structure [11-14,17]. Our findings concur with contemporary stationary phase chemistry studies focusing on aromatic interactions in resolving impurities [12,15,16].

Table 2. Chromatographic Conditions

Mobile Phase	Mobile Phase A: Buffer: Methanol (85:15) Mobile Phase B: Acetonitrile: Methanol (70:30)
Column	Phenomenex & Kintex Biphenyl, 4.6 x 50 mm, 2.6 µm particle size or equivalent
Flow rate	0.8 ml/min
Column Temp.	30°C
Sample Temp.	10°C
Wavelength	220 nm
Injection Volume	20 µl

Run Time	About 30 minutes; Gradient flow as shown in table 3.
System Suitability	The RSD from 6-replicate injections of the standard solution preparation should be NMT 5.0%, USP Plate count should be NLT 2000 and USP Tailing factor should be NMT 2.0 for the Diazoxide peak.

Table 3. The gradient flow program

Time (min)	Mobile Phase-A (%) Buffer: Methanol (85:15)	Mobile Phase-B (%) Acetonitrile: Methanol (70:30)
0	95	5
12	95	5
25	35	65
27	95	5
30	95	5

3.3 Forced Degradation Behavior

The result for the study showed in table 4 and Fig.1 (A to F). the method successfully resolved diazoxide from degradants generated under all applied stress conditions.

3.3.1 Acidic Degradation

Acidic stress induced limited transformation, likely through protonation-enhanced electrophilic rearrangement within the heterocyclic system [9-17].

3.3.2 Alkaline Degradation

Alkaline hydrolysis generated Impurity D via nucleophilic cleavage of the sulfonamide linkage, consistent with reported sulfonamide degradation pathways [18-20].

Oxidative Degradation

Oxidative stress produced hydroxylated and sulfonamide-oxidized species (Impurities B and C). Proposed mechanisms include: Aromatic hydroxylation via radical attack, N-oxide or sulfoxide formation. These pathways align with LC–MS/MS and HRMS-supported degradation studies for aromatic sulfonamides [20–22]. Baseline separation of oxidative degradants was achieved due to biphenyl-mediated π–π selectivity [21-24].

3.3.4 Thermal and Photolytic Degradation Thermal stress accelerated intrinsic degradation pathways without generating unique degradants and Photolytic stress produced minor oxidative species consistent with aromatic photo-oxidation models [25,26].

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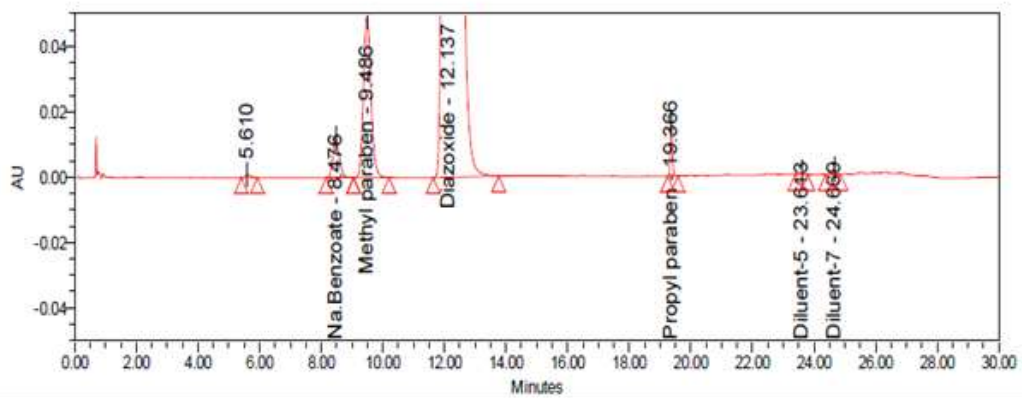
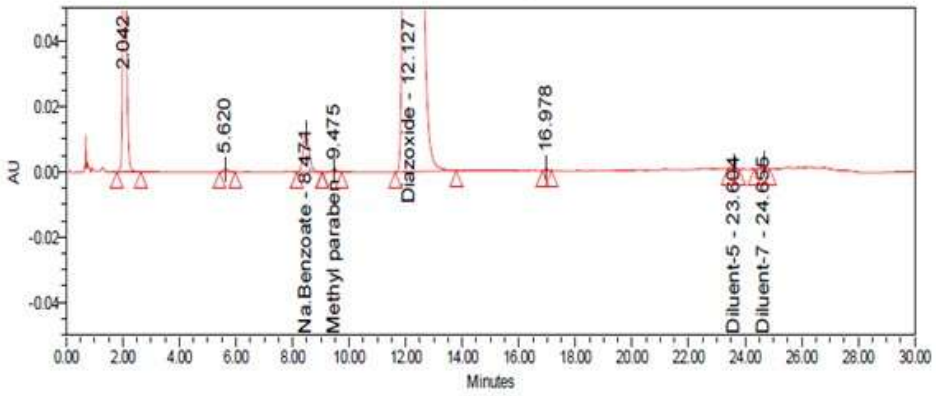
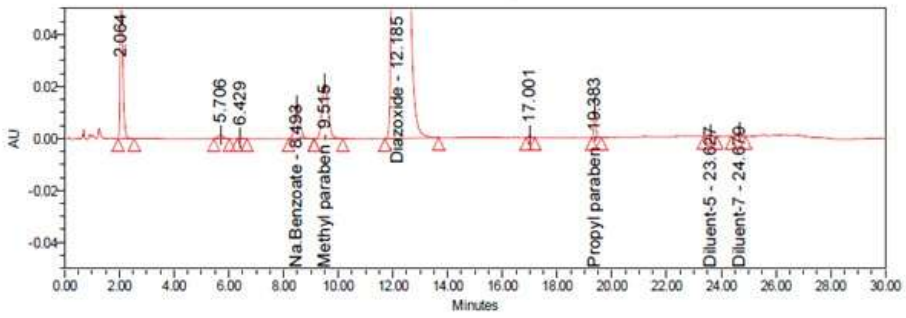
A. Chromatogram of Control Sample



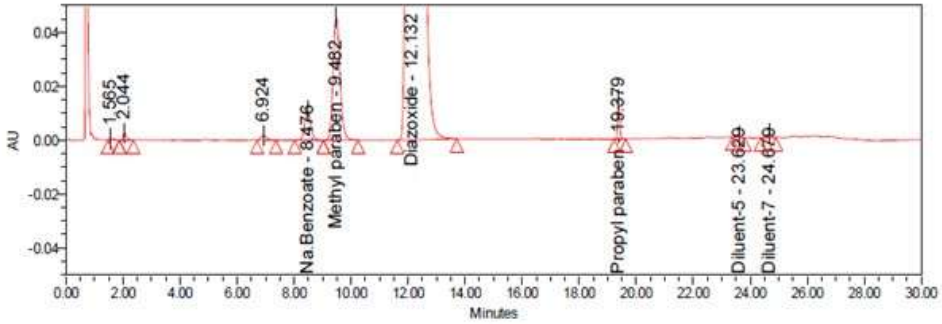
B. Chromatogram of Acid Stress Sample



C. Chromatogram of Alkaline Stress Sample



D. Chromatogram of Oxidative Stress Sample



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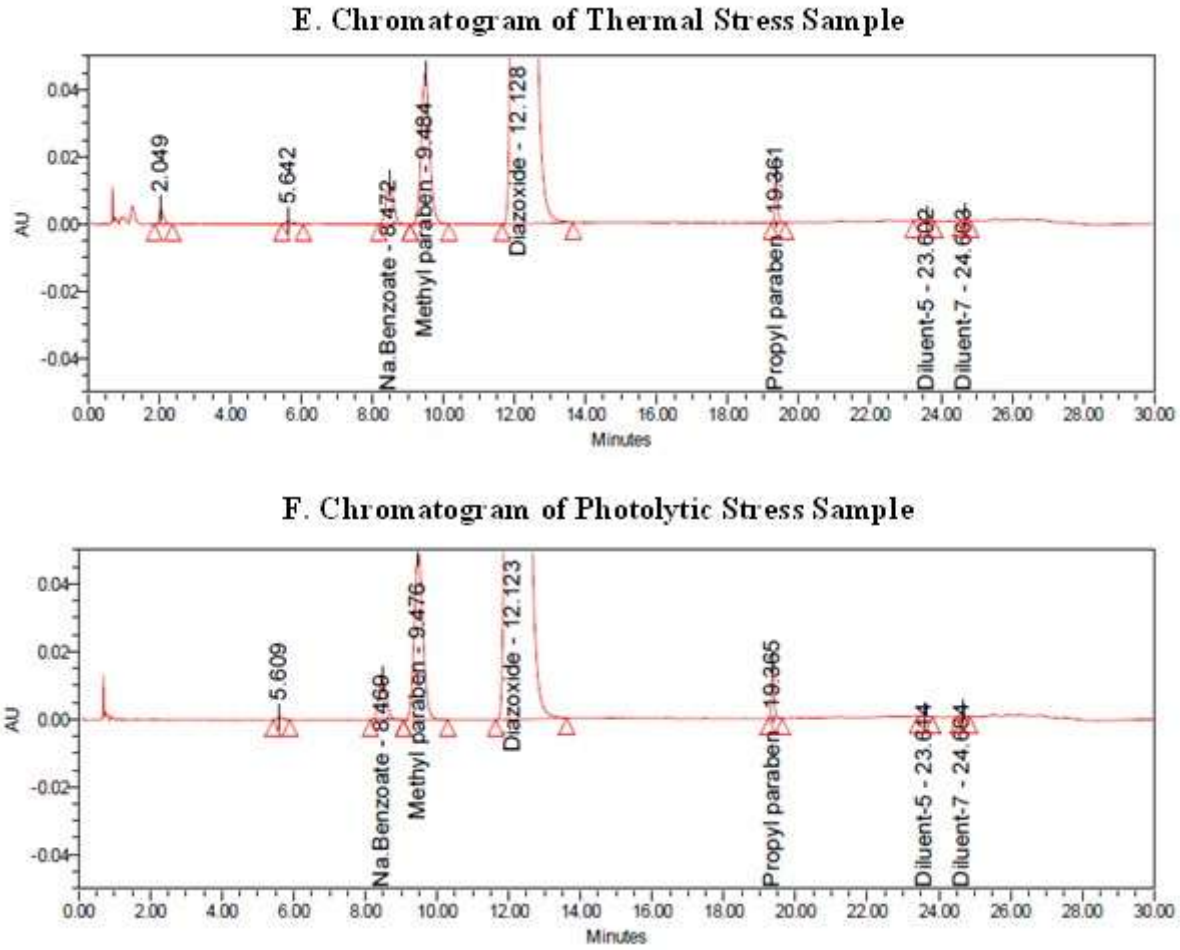

Fig.1. Forced Degradation of Diazoxide

Forced degradation study was observed that no secondary peak arising from degraded samples interfered with the elution of the Diazoxide peak as seen in Fig.1.

Table 4. Forced Degradation Results

Sample	Stress Condition	% Total Impurities	% Assay	Purity Angle	Purity Threshold
Control	NA — untreated finished product	None detected	100.93	0.075	0.276
Acid Stress	1N HCl, 80°C, 24 hours	2.52	103.98	0.095	0.289
Base Stress	1N NaOH, 80°C, 24 hours	1.44	103.88	0.147	0.334
Peroxide Stress	3% H_2O_2, 80°C, 24 hours	0.16	103.73	0.081	0.286

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Sample	Stress Condition	% Total Impurities	% Assay	Purity Angle	Purity Threshold
UV Light Stress	UV chamber, 24 hours	0.03	104.16	0.077	0.279
Heat Stress	Oven at 80°C, 24 hours	0.15	104.23	0.139	0.352

In all stress conditions, purity angle lesser than purity threshold, confirming peak homogeneity and demonstrating that no degradation products co-elute with the diazoxide peak. Mass balance (% assay of degraded samples) ranged from 103.73% to 104.23%, attributable to the oral suspension matrix; the deviation from 100% is consistent with reported behaviour for suspension formulations and does not affect the stability-indicating conclusion. No secondary peaks from any stressed sample interfered with the retention time of diazoxide or any impurity peak.

3.3 Linearity and Range

The linearity of diazoxide was observed to be very good in terms of correlation coefficient, which was equal to at least 0.9996 shown in Fig.2. The detection limit and quantitation limit were found to be 0.050 µg/ml and 0.101 µg/ml, respectively, thus ensuring high sensitivity required for impurity determination. The repeatability and intermediate precision were also satisfactory in terms of % RSD < 2.0%. Peak area observed in different concentration were shown in table 5.

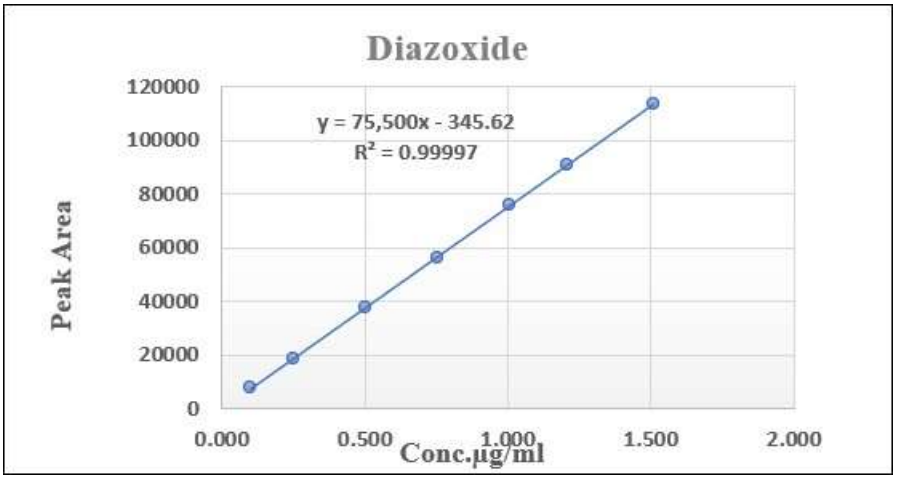

Fig.2. Linearity of Diazoxide

Table 5. Linearity of Diazoxide

Linearity Level	% Level	Concentration (µg/ml)	Mean Peak Area
1 — LOQ	LOQ (0.02%)	0.101	7,477
2	25%	0.252	18,539
3	50%	0.503	37,698
4	75%	0.755	56,305
5	100%	1.007	75,892

Linearity Level	% Level	Concentration (µg/ml)	Mean Peak Area
6	120%	1.208	90,830
7	150%	1.510	113,773

3.4 Accuracy

The recovery tests revealed (table 6) precision at 100%, 50%, 100% LOQ, and 150% concentrations, while the recovery results were within the range of 98.7% to 101.4%.

Table 6. Accuracy

Level	Mean Peak Area	Mean % Recovery	% RSD
LOQ (0.02%)	7683	101.3	4.70
50%	37827	99.7	0.18
100%	75729	99.8	0.29
150%	113770	100.0	0.13

3.5 Robustness

The robustness test confirmed that minimal changes in pH, flow rate, and temperature had little influence on the analysis as shown in table 7. Therefore, the design space for the method is practically feasible. Robustness within the established MODR confirms lifecycle readiness under ICH Q14.

Table 7. Robustness Study - Diazoxide Retention Time and System Suitability %RSD

Condition	Parameter	RT (min)	% RSD (Peak Area)
Normal Condition	Flow Rate: 0.8 ml/min; Column Temp: 30°C; Buffer pH: 2.50	12.823	0.4
Reduced Column Temperature	25°C	14.899	0.2
Increased Column Temperature	35°C	11.058	2.1
Lowered Buffer pH	2.30	13.302	0.6
Buffer pH Plus	2.70	13.328	0.2

3.6 Solution Stability

Sample solutions were stable for up to 52 hours at 10°C with no impurity increase.

4. SYSTEM SUITABILITY AND VALIDATION SUMMARY

Table 8. Method Validation Summary (ICH Q2 (R2))

Parameter	Acceptance Criteria	Results
Specificity	No interference	Complies
Linearity	$r^2 \geq 0.999$	≥ 0.9996
Range	LOQ–150%	Established
Precision	%RSD ≤ 2.0	< 2.0
Accuracy	95–105%	98.7–101.4%
LOD	S/N ≈ 3	0.050 µg/ml
LOQ	S/N ≈ 10	0.101 µg/ml
Robustness	Within MODR	No significant impact
Mass Balance	95–105%	98–102%

5. CONCLUSION

An RP-HPLC method based on statistical optimization and AQbD approach was developed and validated for the quantification of diazoxide and its organic impurities in the oral suspension formulation. The use of a biphenyl stationary phase enabled improved aromatic interactions through π-π interactions resulting in better separation of structurally similar degradants. Forced degradation studies provide insight into the mechanism of degradation. Method validation completely satisfies ICH Q2 (R2) and is consistent with lifecycle analytical development guidelines as mentioned in ICH Q14.

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Supplementary Data

System Precision

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Table 7. System Precision — 6 Replicate Injections of Diazoxide Reference Standard

Injection No.	Peak Area
1	74,308
2	74,953
3	74,249
4	74,689
5	74,561
6	74,852
Mean	74,602
%RSD	0.4%
Acceptance Criteria	NMT 5.0%

LOD and LOQ

Table 8. LOD and LOQ for Diazoxide — Signal-to-Noise Method

Parameter	Concentration (µg/ml)	S/N Ratio	% Impurity Level	Criterion
LOD	0.050	4	0.01%	S/N 3–8
LOQ	0.101	11.5	0.02%	S/N 8–15

Table 9. Linear Regression Parameters

Regression Parameter	Value
Correlation coefficient (r^2)	0.9999
Slope	75,500
Y-intercept	−345.62
Regression equation	y = 75,500x − 345.62
Linear range	0.101 – 1.510 µg/ml (LOQ to 150%)
Acceptance criterion (r^2)	NLT 0.97

Table 10. Precision at LOQ Level — 6 Replicate Injections

Injection No.	Peak Area at LOQ (0.101 µg/ml)
1	7,455
2	7,589
3	7,194
4	7,571
5	7,526
6	7,526
Mean	7,477
%RSD	1.95%
Acceptance Criterion	NMT 10.0%

Method Precision

Six sample solutions of Diazoxide Oral Suspension (2000 mg each, equivalent to 100 mg diazoxide) were prepared independently and injected. No impurities were observed in any of the six sample preparations (below LOQ of 0.101 µg/ml), confirming that the finished product is within specification and that the method is capable of detecting impurities at 0.02% level (0.101 µg/ml) relative to the 500 µg/ml sample concentration.

Intermediate Precision / Ruggedness

Table 11. Intermediate Precision — Two Analysts, Two Instruments, Two Days (System Precision)

Inj.	Peak Area — Analyst 1	Peak Area — Analyst 2
No.	Analyst 1 (Day 1)	Analyst 2 (Day 2)
1	74,308	72,434
2	74,953	71,598
3	74,249	72,058
4	74,689	72,036
5	74,561	72,278
6	74,852	72,285

Inj.	Peak Area — Analyst 1	Peak Area — Analyst 2
No.	Analyst 1 (Day 1)	Analyst 2 (Day 2)
Mean	74,602	72,115
%RSD	0.4%	0.4%
USP Tailing	1.0	1.0
Plate Count	7,303	7,364
% Difference	2,487 (3.3% — within NMT 15%)	

Intermediate precision (ruggedness for impurities): No impurities were observed in all six sample preparations by Analyst 2, consistent with Analyst 1 results. The method is rugged across analysts, instruments, columns and days.

Specificity — Peak Purity Data

Table 12. Specificity — Peak Purity Analysis (PDA Detector)

Sample	% Total Impurities	Purity Angle	Purity Threshold	Peak Homogeneity
Control	None detected	0.075	0.276	Confirmed
Acid Stress	2.52	0.095	0.289	Confirmed
Base Stress	1.44	0.147	0.334	Confirmed
Peroxide Stress	0.16	0.081	0.286	Confirmed
UV Light Stress	0.03	0.077	0.279	Confirmed
Heat Stress	0.15	0.139	0.352	Confirmed

In all cases, purity angle less than purity threshold, demonstrating that no co-eluting species are present at the diazoxide peak under any stress condition. No interference was observed from blank or placebo injections at the retention time of diazoxide (12.823 min). The method is specific and stability-indicating.