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QUALITY BY DESIGN APPROACH FOR DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING RP-HPLC METHOD FOR FINERENONE IN PURE AND BULK FOR OF DRUG

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ABSTRACT

Objectives: Chronic kidney disease associated with type 2 diabetes mellitus poses a significant global cardiorenal burden, necessitating reliable analytical tools for quality assurance of finerenone, a third-generation non-steroidal mineralocorticoid receptor antagonist. The present study aimed to develop and validate a stability-indicating reversed-phase high-performance liquid chromatography method for quantitative determination of finerenone in pure and bulk drug form using an Analytical Quality by Design framework, ensuring regulatory compliance and clinical-grade analytical reliability. **Methods:** Physicochemical characterization encompassing UV absorption maxima, melting point, and solubility profiling was performed. A 3^2 full factorial design with ethanol proportion and flow rate as critical method parameters was employed for systematic chromatographic optimization. The Method Operable Design Region was established through response surface modeling. Method validation was conducted per ICH Q2(R2) guidelines evaluating specificity, linearity, accuracy, precision, sensitivity, robustness, solution stability, and filter compatibility. **Results:** The absorption maximum was identified at 239 nm. The desirability-based optimized batch (Run 5: 40% ethanol, 1.0 mL/min, desirability = 1) yielded retention time of 3.12 min and tailing factor of 1.18. Linearity was demonstrated over 10–120 µg/mL (r^2 = 0.9998). LOD and LOQ were 0.026 µg/mL and 0.078 µg/mL respectively. Mean accuracy was 99.99 ± 0.25% and precision %RSD remained below 0.30%. Formulation assay of Kerendia® tablets yielded 99.97 ± 0.21% label claim. **Conclusion:** The validated AQbD-guided, ethanol-based, MS-compatible method demonstrated superior sensitivity, robustness, and stability-indicating capability, offering significant practical implications for routine quality control.

KEYWORDS: Finerenone; RP-HPLC; Analytical Quality by Design; Stability-indicating method; ICH Q2(R2); Method Operable Design Region; Factorial design; Cardiorenal.

INTRODUCTION

The cardiorenal catastrophic situation of the type 2 diabetes mellitus is one of the most urgent twenty first century crises [1]. Worldwide, more than 536 million adults have type 2 diabetes and almost half of these patients will develop a complication of diabetes diabetic kidney disease which is a major cause of end-stage renal disease, cardiovascular illness and premature death [2]. Based on epidemiological projections, by 2040 CKD could become one of the five leading causes of early death, driving an unsustainable economic burden on the healthcare system, and annual costs of cardiovascular-related activity could be more than 350 billion dollars in the USA alone. But despite the availability of effective renin-angiotensin-aldosterone system blockade, the likelihood of disease progression remains large and can be explained by the fact that mineralocorticoid receptor signaling causes inflammation and fibrosis, which is not completely controlled [3]. As all mineralocorticoid receptor antagonists, traditional ones, such as spironolactone and eplerenone, have incidences of hyperkalemia of up to 19–24% in CKD patients, and they have very common effects on antiandrogens, severely limiting their clinical utility [4]. The limitations, together with the growing global occurrence of cardiorenal comorbidities, underscore the need for newer and safer therapeutic agents with suitable powerful analytical platforms, fully accepted by regulatory authorities and providing conformance from pre-development through to clinical use, ensuring a consistent drug quality [5].

Bayer AG's third-generation non-steroidal mineralocorticoid receptor antagonist Finerenone, approved by the FDA in July 2021, overcomes the vital shortcomings of preceding steroidal MRAs [6]. Finerenone is chemically named 4-(4-cyano-2-methoxyphenyl)-5-ethoxy-2,8-dimethyl-1,4-dihydro-1,6-naphthyridine-3-carboxamide, has a molecular formula of $C_{21}H_{22}N_4O_3$ and has a molecular weight of 378.43 g/mol. The dihydropyridine framework is bulky, fully occupying the mineralocorticoid receptor (MR) ligand-binding domain (LBD) and not activating it, allowing it to suppress activation of MR without its interaction with androgen, estrogen, progesterone, or glucocorticoid receptor-LBDs (LRBDs), which significantly lowers off-target toxicity [7]. The anti-inflammatory and anti-fibrotic activity of Finerenone is extremely high in renal and cardiac tissues through the inhibition of aldosterone and cortisol mediated downstream transcriptional activation of pro-inflammatory and pro-fibrotic genes [8]. Landmark clinical trials, for both FIDELIO-DKD and FIGARO-DKD, have shown that finerenone improved composite kidney and cardiovascular outcome in comparison to placebo, and had a significantly improved hyperkalemia profile compared with the steroidal antagonists [9]. Yet, although it is becoming more widely implemented in the clinic, well established and validated analytical methods for the routine quantification of this drug in clinical

practice are still few and far between in the literature, thus establishing a high unmet need in the pharmaceutical quality assurance field [10].

The Analytical Quality by Design approach developed in the present work and now internationally recognised according to ICH Q14 agreement and harmonised with ICH-Q2(R2) (IQ 2013), is used to fill this analytical void. The AQbD approach is fundamentally different from one-factor-at-a-time method development, as it starts from a clear definition of an Analytical Target Profile, a risk assessment process driven by Ishikawa method to identify critical method parameters and a 3^2 full factorial design of experiments used to systematically participate the multivariate relationship between chromatographic factors and responses [11]. Together with a volatile 10 mM ammonium formate buffer (pH 3.5), the resulting mobile phase system proved to be MS-compatible, sustainable, and chromatographically very good when compared to acetonitrile and methanol, which are less environment friendly and greener. As per the philosophy of QbD [12], the Method Operable Design Region suggested by response surface modelling was determined such a way that the method would be consistent, with satisfactory performance even if the operations are slightly varied. An optimized method was shown to be suitable for these analysis purposes by performing forced degradation experiments under acidic, alkaline, oxidative, and thermal, photolytic and neutral stress conditions [13].

In the present study, a quantitative method for finerenone has been developed and validated by system QbD approach in pure and bulk drug form by RP-HPLC. Specific aims are physicochemical characterization of the drug, chromatographic optimization as per DoE & establishment of Method Operable Design region, comprehensive ICH Q2(R2) validation including specificity, linearity, accuracy, precision, sensitivity, robustness and formulation assay.

MATERIALS AND METHODS

MATERIALS

Finerenone working standard (99.8% purity) was obtained from Sigma-Aldrich India, Merck Life Science Pvt. Ltd., Bangalore, India and Yarrow Chem Products, Mumbai, India were used for bulk drug material. Drug was obtained in the form of tablets (10mg) and called Kerendia® (Bayer Zydus Pharma Pvt. Ltd.). HPLC-quality ethanol, acetonitrile and methanol were purchased from Qualigens Fine Chemicals, Thermo Fisher Scientific India Pvt. Ltd., Mumbai, India. The ammonium formate, ammonium acetate, potassium dihydrogen phosphate, orthophosphoric acid (all HPLC grade) were obtained from the Spectrochem Pvt. Ltd. (Mumbai, India). Hydrochloric acid of strength 0.1 N (Rankem), RFCL Ltd. (New Delhi, India), sodium hydroxide of strength 0.1 N (Rankem) and hydrogen peroxide (30%w/w) (Merck Life Science Pvt. Ltd. Mumbai India) were purchased. HPLC-grade water was prepared in house using a Milli-Q purification system (Merck Millipore, Bangalore, India) and nylon membrane filters (0.45 µm) were obtained from Pall India Pvt. Ltd. (Mumbai, India). Any chemicals and reagents not used were all of analytical grade.

METHODS

Determination of Absorption Maxima (λ_{max})

Finerenone required its UV absorption maximum (λ_{max}) was determined to select an appropriate wavelength for chromi-tographic measurement. A stock solution (1000µg/mL) was made by dissolving 10mg of the finerenone working standard in a 10mL volumetric flask into HPLC-grade ethanol and diluting this to 10µg/mL with the mobile phase (ethanol: 10mM ammonium formate pH3.5). The solution was scanned within 200–400 nm range by using a double beam UV-Visible Spectpophotometp (Model LMSPUV1000B, Labman Scientific Instruments Pvt. Ltd, Chennai, India) with the mobile phase as blank. The wavelength of the maximum absorbance values were determined and used for all later chromatographic activities (n = 3) [14,15].

Melting Point Determination

To confirm drug identification and purity, melting point of finerenone bulk drug was determined with a calibrated digital melting point apparatus (Veego Instruments Corporation, Mumbai, India). The finely powdered drug was packed in a closed capillary tube, heated at a controlled rate 2°C/min and the final and starting temperatures recorded which were compared with the standard expected range of 195-197°C. The results were presented as mean ± standard deviation (n=3) [16,17].

Solubility Study

Finerenone was characterized to be in equilibrium solubility in solvents with different polarity by the solvent saturation method. A large excess of the drugs was added to water, 0.1 N HCl, 0.1 N NaOH, phosphate buffer pH 6.8, ethanol, methanol, acetonitrile, chloroform and DMSO kept in stoppered glass vials and allowed to stand on a mechanical shaker (Remi Elektrotechnik Ltd., Mumbai, India) at 25 ± 0.5°C for 24 hours. The filtered samples (filtered on nylon membrane, 0.45 µm) were properly diluted and analyzed by spectrophotometric analysis using the above-mentioned range of λ_{max} set for each band on the instrument (Model no: LMSPUV1000 B, Labman Scientific Instruments Pvt. Ltd., Chennai, India). Expressing solubility as mg/mL and interpreting it as per Indian Pharmacopoeia descriptors. Values are reported as mean ± SD (n=3) [18].

Preparation of Standard and Sample Solutions

A stock solution of a working standard of finerenone (1000 µg/mL) was prepared by accurately weighing and dissolving 10 mg of the drug in HPLC-grade ethanol, briefly sonicated for 5 minutes and stored under refrigerated conditions (4 °C) and in the dark. Mobile phase being optimized etanol : 10 mM ammonium formate buffer, pH 3.5, was used to make standard solutions within the range of concentration (10–100 µg/mL) by serial dilution for linearity and for constructing the calibration curve. Finerenone bulk drug was similarly prepared for sample solution (1000 µg/mL) which was diluted to 100 µg/mL working solutions with the mobile phase before injecting. The solutions were all freshly prepared on the day of analysis and filtered through a 0.45 µm nylon membrane filter before injection [19].

Experimental Design

A 3^2 full factorial design was employed for systematic QbD-based optimization of the RP-HPLC method. Ethanol proportion in the mobile phase (X_1) and flow rate (X_2) were determined as the critical method parameters identified by a risk assessment using an Ishikawa cause and effect diagram, and were subjected to a three-level experiment with each parameter yielding 9 experimental runs that were randomized. Retention time (Y_1) and tailing factor (Y_2) were chosen as the dependent variables. The other chromatographic parameters: Inertsil ODS-3V C18 (250 mm × 4.6 mm i.d., 5 µm), the temperature of the column was 30°C, the pH of the 10 mM ammonium formate buffer was 3.5, the wavelength in the detector was measured at 252 nm, volume of injection was 20 µL and run time was 10 minutes. The second order polynomial was used to fit response data: $Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_{12}X_1X_2 + \beta_{11}X_1^2 + \beta_{22}X_2^2$ The ANOVA statistical analysis was used to determine the different parameters used, while the Method Operable Design Region (MODR) was set up by response surface and contour plots. According to the desirability function and plot of overlay, all further validation study was performed under the optimal conditions of ethanol : 10 mM ammonium formate buffer (pH 3.5): 40:60 (v/v) and flow rate 1.0 mL/min at 30 °C [20].

Table 1: Independent and Dependent Variables in 3^2 Full Factorial Design.

Independent Variables	**Levels**		
	Low (−1)	**Medium (0)**	**High (+1)**
X_1: Ethanol proportion (% v/v)	35	40	45
X_2: Flow rate (mL/min)	0.8	1.0	1.2
Dependent variables			**Goal**
Y_1: Retention time (min)			In range (2.0 – 5.0 min)
Y_2: Tailing factor			Target ≤ 2.0

Table 2: 3^2 Full Factorial Design Nine Experimental Runs.

Run	**X_1 (Ethanol %)**	**X_2 (Flow rate mL/min)**
1	35	0.8
2	35	1.0
3	35	1.2
4	40	0.8
5	40	1.0
6	40	1.2
7	45	0.8
8	45	1.0
9	45	1.2

Execution of DoE Experimental Runs

A working solution of finerenone from the standard stock solution was prepared by HPLC grade ethanol to a concentration of 1000 µg/mL and then diluted to 100 µg/mL with the optimized mobile phase prior to injection, which was then filtered using nylon 0.45 µm MEMBRANE of HPLC grade ethanol. Ethanol proportion (X_1) and flow rate (X_2) were tested at three coded levels (−1, 0, +1), and

nine experimental runs of the 3^2 full factorial design were performed by using the coded levels of HPLC system (Shimadzu LC-20AD, Shimadzu Analytical India Pvt. Ltd., Mumbai, India), with a UV-Visible detector (SPD-20A). The retention time (Y_1) and tailing factor (Y_2) were measured for each run and analysed statistically [21].

Method Validation

The developed RP-HPLC method was validated in agreement with the ICH guidelines which included system suitability, specificity, peak purity, forced degradation, linearity, LOD, LOQ, accuracy, precision, robustness, solution stability and filter compatibility [22].

System Suitability

The suitability of this system was determined by six injections (n=6) of freshly prepared working standard finerenone under optimized conditions. Retention time, Tailing factor, The theoretical plates, and %RSD were assessed whether it meets the ICH Q2(R2) and USP criteria which are Tailing factor ≤ 2.0, Theoretical plates ≥ 2000, %RSD $\leq 2.0\%$ [23].

Specificity and Peak Purity

Optimized conditions were compared between blank, standard and sample using chromatograms to establish specificity. Purity was evaluated by the evaluation of the UV spectra at the peak start, peak apex and peak end by using a photodiode array detector, and if the purity angle was lower than the purity threshold value, it was considered as signal was from the peak which was free of co-eluting impurities (n=3) [24].

Forced Degradation Studies

Six stress conditions were used for forced degradation studies, based on ICH Q1A(R2) guidelines. The finerenone solutions were made up to 0.1N hydrochloric acid and 0.1N sodium hydroxide solutions and refluxed at 60°C. for 1 hour, after which they were neutralized before being analysed. Oxidative degradation was carried out at 25°C for 1 hour at the rate of 3% (v/v) H_2O_2. Thermal degradation test was conducted by exposing solid drug to 105°C in hot air oven for 24 hours. Photolytic degradation was done for 24 hrs /ICH Q1B in a photostability chamber under UV (254 nm) and visible light (400-800 nm) condition. To remove the drug from its parent form, neutral hydrolysis was attempted by reflux in HPLC grade water at 60C for 1 hour. In all conditions, an unstressed standard was used as a control. The method was deemed to be stability-indicating when the resolution between finerenone and all the degradation peaks was ≥ 2.0 obtained for n = 3 [25].

Linearity

The optimized mobile phase (see above) was used to isolate the stock solution and produce seven concentrations by serial dilution. Linearity was checked using the different concentrations. Least-squares linear regression was used to evaluate peak areas vs. concentration. The correlation coefficient (r^2), the slope and the intercept were calculated: $r^2 \geq 0.999$ was accepted (n = 7) [26].

Limit of Detection and Limit of Quantification

LOD and LOQ were calculated using the equations:

LOD = 3.3 × σ / S LOQ = 10 × σ / S

where σ is the standard deviation of the y-intercept and S is the calibration curve slope. Values were experimentally verified by signal-to-noise ratios of 3:1 and 10:1 respectively, with %RSD at LOQ ≤ 10% (n=6) [27].

Accuracy

The standard addition method were performed thrice at each 120%, 80% and 100% of the target concentration (n=9) to determine accuracy. Recovery in percentage was determined by:

% Recovery = (Experimentally found concentration / Theoretical concentration) × 100

Acceptance criteria were 98.0–102.0% recovery with %RSD ≤ 2.0% [27].

Precision

The repeatability or intra-day precision was achieved by six replicate injections on a single day with %RSD for the peak area of the results ≤2.0% (ICH Q2(R2) criteria), whereas the inter-day precision or intermediate precision was obtained by six injections over three days with %RSD for each peak area of the results ≤2.0% (ICH Q2(R2) criteria) [28].

Robustness

The robustness was checked by deliberately changing ethanol proportion (by ±2%), buffer pH (by ±0.1 unit) and flow rate (±0.1 mL/min) from optimized conditions, respectively. Under each condition, the system suitability parameters were evaluated; the %RSD for the peak area at the tolerances below was not exceeded (n=3) [29].

Solution Stability

For standard and sample solutions, the stability was evaluated after 0, 6, 12 and 24 hours at room temperature (25 ± 2°C). Peak areas at each time point were compared to a freshly prepared reference standard. Body weight changes were acceptable for Solutes stability (n=3) when they were within 2.0% of the initial value and %RSD [30].

Filter Compatibility

The filter compatibility was verified by comparing the peak areas of the finerenone standard filtered through nylon membrane (0.45 µm) with obtained reference peak areas (centrifuge material, 3000 rpm, 10 minutes) by the same analytical method used for qualitative analysis of samples. To saturate a filter with adsorption sites, initial filtrate volumes were discarded. Filter compatibility (n=3) was ensured with percentage recovery 98.0–102.0%, %RSD ≤ 2.0% [31].

Assay of Pharmaceutical Formulation

Twenty tablet of 'Kerendia®' (10mg, Bayer Zydus Pharma Pvt. Ltd.) were weighed, averaged and finely powdered. Tablet powder equivalent to 10mg finerenone was sonicated in HPLC-grade ethanol for 10 minutes, diluted to 1000 µg/mL, filtered using 0.45 µm nylon membrane and finally diluted in optimum mobile phase to the working concentration. The quantity of drug was determined by:

% Label Claim = (Peak area of sample / Peak area of standard) × (Concentration of standard / Concentration of sample) × (Average tablet weight / Label claim weight) × 100

Acceptance criteria were 98.0–102.0% label claim with %RSD ≤ 2.0% (n=3) [32].

RESULTS AND DISCUSSION

RESULTS

Scanning absorbance maxima

The finerenone showed a well-defined absorption maximum in its UV spectrum at 239 nm as can be seen in the sketch below (Figure 1). The chosen wavelength for all subsequent chromatographic analysis was this wavelength. The observed lambda max was consistent with the chromophoric system of dihydropyridine backbone of finerenone being aromatic and conjugated carbonyl.

Figure 1: Scanning absorbance maxima of Finerenone (239 nm)

Melting point

Melting point of the finerenone bulk drug was determined to be 195.67 ± 0.47°C as presented in the Table 3, which is in the standard range of reported melting point between 195-197°C. The limited melting range with the good agreement to the pharmacopoeial reference established the identity and purity (within acceptable limits) of the bulk drug product obtained for the study, thus ensuring its suitability for subsequent analytical method development.

Table 3: Melting Point Determination of Finerenone Bulk Drug

Sr. No.	Compound	Melting Point Observed (°C)	Standard Melting Point (°C)
1	Finerenone	195.67 ± 0.47	195 – 197

Solubility study

The solubility of finerenone as a function of the polarity of solvents is summarized in Table 4. The drug was observed to be practically insoluble in water (0.024 ± 0.003 mg/mL), sparingly soluble in 0.1N HCl and freely soluble in methanol (287.40 ± 4.62 mg/mL). HPLC grade ethanol was used as the organic modifier, and was found to have good solubility properties (68.30 ± 2.18 mg/mL).

Table 4: Solubility Profile of Finerenone Bulk Drug.

Sr. No.	Solvent	Solubility (mg/mL)	Inference
1	Water	0.024 ± 0.003	Practically insoluble
2	0.1 N Hydrochloric acid (pH 1.2)	14.51 ± 0.42	Sparingly soluble
3	Phosphate buffer (pH 6.8)	0.026 ± 0.002	Practically insoluble
4	0.1 N Sodium hydroxide	0.071 ± 0.005	Very slightly soluble
5	Methanol	287.40 ± 4.62	Freely soluble
6	Ethanol (HPLC-grade)	68.30 ± 2.18	Soluble

7	Acetonitrile	28.45 ± 1.37	Sparingly soluble
8	Chloroform	54.20 ± 3.11	Soluble
9	Dimethyl sulfoxide (DMSO)	42.60 ± 2.84	Soluble

Results of Chromatograms as per DOE runs

The values of retention time (2.31 to 4.82 min) and tailing factor (1.18 to 1.48) obtained in the nine experimental runs as per 3^2 full-factorial experimental design are listed in table 5. The best chromatographic performance was obtained for run 5 (40% ethanol 1.0 mL/min) which gave a retention time of 3.12 min, tailing factor of 1.18 and a maximum number of theoretical plates of 6842.

Table 5: 3^2 Full Factorial Design Matrix with Observed Chromatographic Responses for Finerenone.

Run	Retention Time (min)	Tailing Factor	Theoretical Plates (N)	Peak Area (mAU·s)
1	4.82	1.48	4124	485632
2	3.91	1.42	4386	512874
3	3.24	1.38	4512	498341
4	4.21	1.28	5214	568742
5	3.12	1.18	6842	624563
6	2.68	1.22	6124	598217
7	3.54	1.35	5384	542186
8	2.74	1.24	5876	574932
9	2.31	1.19	5142	521468

Optimization

Retention Time (Y_1)

The linearity model gave the closest fit to the data of retention time and was given a strong fit with an adjusted R^2 and predicted R^2 of 0.9603, and 0.9375, respectively (Table 8.5). The overall linear model was highly significant ($F = 97.70$, $P = 2.64 \times 10^{-5}$) and there was no significant lack of fit. Ethanol proportion (A: $F = 73.77$, $p = 1.37 \times 10^{-4}$) and flow rate (B: $F = 121.62$, $p = 3.31 \times 10^{-5}$) were found to be statistically significant for the retention time and flow rate had relatively larger effect (Table 8.6). The resultant polynomial equation is: $Y_1 = 3.397 - 0.563A - 0.723B$ The negative sign for both the factors validate the decrease in retention time for added ethanol proportion and flow rate, with the larger factor (B of –0.723) showing greater effect of flow rate. The contour plot (Figure 8.20) and the three dimensional response surface plot (Figure 8.21) showed consistent smooth monotonically decreasing surfaces over the space size. The maximum retention time was concluded at low ethanol (35%) and low flow rate (0.8 mL/min) while the minimum retention time was found at high ethanol (45%) and high flow rate (1.2 mL/min). The central design point (40% ethanol and 1.0 mL/min) showed a retention time of 3.12 minutes in the range of 2.0–5.0 minutes as desired.

Tailing Factor (Y_2)

The fit summary showed that the fit of the quadratic model is fairly better than both linear (adjusted R 2 = 0.5140) and two-factor interaction model with adjusted R 2 = 0.9170 and R 2 predicted = 0.6775. The results indicated that overall model was significant (p = 0.0181) but further analysis by ANOVA revealed both ethanol proportion (p = 0.0071) and flow rate (p = 0.0244) as significant linear non-curvilinear terms, while ethanol proportion squared (A2, p = 0.0129) was significant curvilinear non-curvilinear terms of the model. There were no significant interaction term of AB or quadratic term of B^2 (Table 8.8). The resulting polynomial equation was: $Y_2 = 1.202 - 0.0833A - 0.0533B - 0.015AB + 0.1167A^2 + 0.0367B^2$ The negative linear coefficients of A and B showed a decrease in the tailing factor as the concentration of the factors increased in the beginning; the positive coefficient of A^2 (+0.1167) verified a curvilinear response in which the lowest level of tailing factor occurred at an intermediate proportion of the ethanol and the highest concentrations of ethanol resulted in a greater tailing factor. These behaviors were seen in the contour plot (Figure 8.24) and response surface three-dimensional plot (Figure 8.25) with minimum tailing factor (1.18) at 40% ethanol and 1.0 mL/min, while the highest values, >1.40 were observed, at the extreme low ethanol level of 35% with low flow rate; this optimum solution was found at the center of the Method Operable Design Region.

Table 6. Fit summary for chromatographic responses of the 3^2 full factorial design for RP-HPLC method optimization of finerenone.

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	Remark
Retention Time (Y_1)					
Linear	2.644×10^{-5}	—	0.9603	0.9375	Suggested
Tailing Factor (Y_2)					
Quadratic	0.0260	—	0.9170	0.6775	Suggested

Table 7. ANOVA summary for the selected models for chromatographic responses of finerenone RP-HPLC method optimization.

Source	Sum of Squares	df	Mean Square	F-value	p-value	Significance
Retention Time (Y_1)						
Model	5.0433	2	2.5217	97.70	2.644×10^{-5}	Significant
A – Ethanol Proportion	1.9041	1	1.9041	73.77	1.369×10^{-4}	
B – Flow Rate	3.1393	1	3.1393	121.62	3.306×10^{-5}	
Residual	0.1549	6	0.0258	—	—	
Cor Total	5.1982	8	—	—	—	
Tailing Factor (Y_2)						
Model	0.08954	5	0.01791	18.67	0.0181	Significant
A – Ethanol Proportion	0.04167	1	0.04167	43.44	0.0071	
B – Flow Rate	0.01707	1	0.01707	17.79	0.0244	

AB	0.00090	1	0.00090	0.938	0.4042	
A^2	0.02722	1	0.02722	28.38	0.0129	
B^2	0.00269	1	0.00269	2.803	0.1927	
Residual	0.00288	3	0.00096	—	—	
Cor Total	0.09242	8	—	—	—	

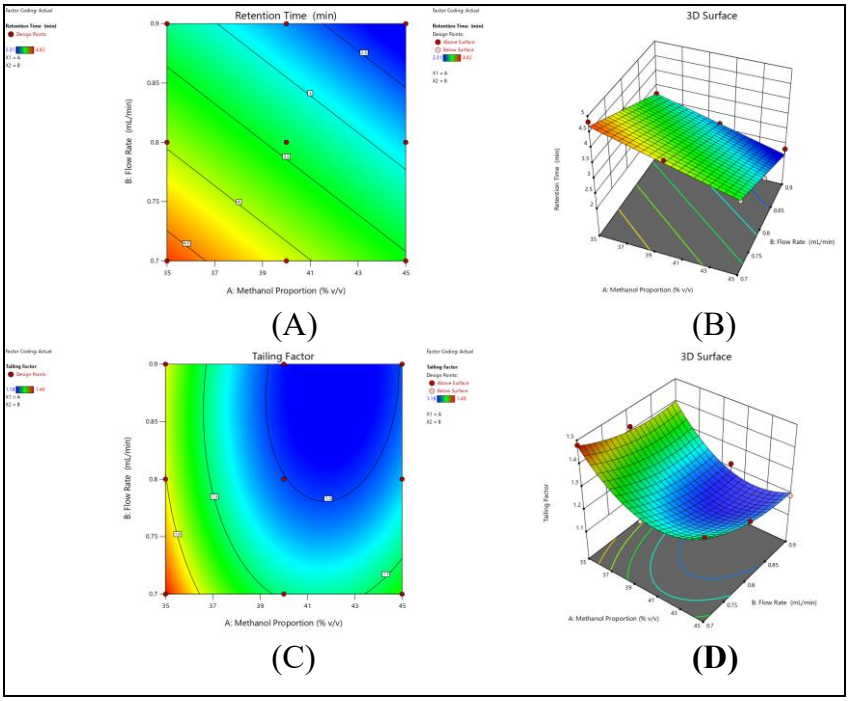

Figure 2. Influence of ethanol proportion and flow rate on chromatographic performance of finerenone in the 3^2 full factorial design optimization. (A) Two-dimensional contour plot and (B) three-dimensional response surface plot depicting the combined effect on retention time (Y_1); (C) two-dimensional contour plot and (D) three-dimensional response surface plot depicting the combined effect on tailing factor (Y_2).

Figure 3: RP-HPLC chromatogram of Optimized Batch

Validation of statistical model

The predicted chromatographic responses were compared with the experimental responses obtained by the target chromatographic variables at the target conditions (Table 8) and gave a good agreement that verifies the validity of the predictive accuracy of the optimized polynomial models. The experimental retention time (3.12 min) and tailing factor (1.18) established relative errors of 2.88% and 0.51% respectively when compared with the predicted value with a global desirability of 1, thus showing the reliability and robustness of the models obtained by the DoE in method optimization.

Table 8: Validation of Optimized Chromatographic Conditions by Comparison of Predicted and Experimental Values of Responses.

Run	Response	Predicted value	Experimental value	% Relative Error	Desirabilitry
5	Retention time	3.21	3.12	2.88	1
	Tailing factor	1.174	1.18	0.51	

Results of Method Validation

System Suitability

The system suitability parameters obtained for successive injections under optimum conditions are shown in Table 9. Mean retention time, peak area, tailing factor, and theoretical plates were recorded as 3.12 ± 0.007 min, 624417 ± 473 mAU·s, 1.18 ± 0.007, and 6845 ± 19.4, respectively. The %RSD values were kept below 2.0%, tailing factor was found to be less than 2.0 and theoretical plates more than 2000 indicating good system performance in the range before validation.

Table 9: System Suitability Parameters of Optimized RP-HPLC Method for Finerenone.

Sr. No.	Parameter	Injection 1	Injection 2	Injection 3	Injection 4	Injection 5	Injection 6	Mean ± SD	%RSD	Acceptance Criteria
1	Retention Time (min)	3.12	3.11	3.13	3.12	3.11	3.12	3.12 ± 0.007	0.23	≤ 2.0%
2	Peak Area (mAU·s)	624563	623841	625124	624287	623954	624731	624417 ± 473	0.08	≤ 2.0%
3	Tailing Factor	1.18	1.19	1.18	1.17	1.19	1.18	1.18 ± 0.007	0.59	≤ 2.0
4	Theoretical Plates (N)	6842	6818	6874	6831	6857	6849	6845 ± 19.4	0.28	≥ 2000

Specificity and Peak Purity

The specificity-peak purity results are presented in Table 10. No peak appeared in the blank mobile phase at the same retention time where the peak of finerenone appeared, indicating no interference from the blank mobile phase. Both normal and bulk drug sample solutions gave a single sharp and symmetrical peak at 3.12 min, with peak purity angles (0.142 and 0.156 were less than the peak purity angles limit (0.387 and 0.412) indicating spectral homogeneity and specificity of the method.

Table 10: Specificity and Peak Purity Results for Finerenone.

Sr. No.	Solution	Retention Time (min)	Peak Observed	Interference at Rt of Finerenone	Peak Purity Angle	Peak Purity Threshold	Inference
1	Blank (Mobile Phase)	—	None	Absent	—	—	No interference
2	Standard Solution	3.12	Single sharp peak	Absent	0.142	0.387	Pure
3	Bulk Drug Sample	3.12	Single sharp peak	Absent	0.156	0.412	Pure

Forced Degradation Studies

The results obtained from the forced degradation tests are reported in Table 11. Finerenone showed to be susceptible to acid hydrolysis (84.32% remaining), alkaline hydrolysis (78.54%) and oxidative hydrolysis (81.74%) giving rise to 2-3 well-resolved degradation peaks with resolution values higher than 2.0 in each case. Minor degradation was observed under the thermal and photolytic conditions whereas negligible degradation (96.82%) was observed under the neutral hydrolysis condition. The good peak purity of the main finerenone peak was retained in all stress conditions proving a stress stability that corresponds to the stability indicating capability of the method.

Table 11: Results of Forced Degradation Studies of Finerenone

Sr. No.	Stress Condition	Stress Reagent / Condition	% Drug Remaining	No. of Degradation Peaks	Resolution from Main Peak	Peak Purity	Inference
1	Acid Hydrolysis	0.1 N HCl, 60°C, 1 hr	84.32 ± 0.42	2	3.84, 4.12	Pure	Partially degraded
2	Alkaline Hydrolysis	0.1 N NaOH, 60°C, 1 hr	78.54 ± 0.63	3	3.21, 4.56, 5.12	Pure	Partially degraded
3	Oxidative Degradation	3% H_2O_2, 25°C, 1 hr	81.74 ± 0.51	2	3.64, 4.87	Pure	Partially degraded
4	Thermal Degradation	105°C, 24 hr (solid state)	92.18 ± 0.38	1	4.24	Pure	Slightly degraded
5	Photolytic Degradation	UV 254 nm + Visible, 24 hr	88.46 ± 0.44	1	3.98	Pure	Slightly degraded
6	Neutral Hydrolysis	Water, 60°C, 1 hr	96.82 ± 0.29	0	—	Pure	Stable
7	Unstressed Control	Mobile phase, ambient	100.00 ± 0.18	0	—	Pure	Stable

Linearity

The optimized RP-HPLC method was found to be linear with concentration at active range of 10–120 µg/mL as shown in Table 12. Peak area had an excellent linear relationship with concentration with correlation coefficient (r2) of 0.9998 and regression equation (y = 6241.8x + 312.4), which exceeded the accepted value of 0.999. The near zero y-intercept and the linear response of the increment areas of all the seven concentration level were established as linearlity and proportional detector response of the method.

Table 12: Linearity Data for Finerenone by Optimized RP-HPLC Method.

Sr. No.	Concentration (µg/mL)	Mean Peak Area ± SD
1	10	62381 ± 138
2	20	124839 ± 193
3	40	249852 ± 263
4	60	374565 ± 278
5	80	499866 ± 247
6	100	624509 ± 643
7	120	749006 ± 341
Regression Equation		y = 6241.8x + 312.4
Correlation Coefficient (r^2)		0.9998
Slope		6241.8
Y-Intercept		312.4

Limit of Detection and Limit of Quantification

The standard deviation of the y-intercept and the slope of the calibration curve were calculated and LOD and LOQ values were determined as 0.026 µg/mL and 0.078 µg/mL, respectively (Table 13). The experimental determination of the two signal to noise ratios (roughly 3:1 and 10:1) gave in both cases very closely concordant values of 0.03 µg/mL and 0.08 µg/mL respectively. The %RSD of the LOQ was found to be 1.24%, hence the method proved to be sensitive with a %RSD of ≤ 10.0% which is accepted by ICH Q2(R2) as an acceptable acceptance criterion.

Table 13: LOD and LOQ of Finerenone by Optimized RP-HPLC Method.

Sr. No.	Parameter	Value	Method of Determination
1	Standard Deviation of Intercept (σ)	48.62	From regression analysis
2	Slope (S)	6241.8	From calibration curve
3	LOD (calculated)	0.026 µg/mL	3.3 × σ / S
4	LOQ (calculated)	0.078 µg/mL	10 × σ / S
5	LOD (experimental, S/N ≈ 3:1)	0.03 µg/mL	Signal-to-noise ratio
6	LOQ (experimental, S/N ≈ 10:1)	0.08 µg/mL	Signal-to-noise ratio
7	%RSD at LOQ (n=6)	1.24%	—
8	Acceptance Criteria (%RSD at LOQ)	≤ 10.0%	ICH Q2(R2)

Accuracy

Table 14 shows the accuracy data obtained by the standard addition method at 80%, 100% and 120% concentrations. Mean percentage recoveries at each level were 99.94 ± 0.29%, 100.02 ± 0.21%, and 100.01 ± 0.24% respectively, with an overall mean recovery of 99.99 ± 0.25%. All individual and mean %RSD values were below 0.30% which is equal with the criteria for accurate optimized method of 98.0–102.0% recovery and %RSD ≤ 2.0%.

Table 14: Accuracy (% Recovery) of Finerenone by Optimized RP-HPLC Method.

Sr. No.	Level	Target Concentration (µg/mL)	Amount Added (µg/mL)	Amount Found (µg/mL)	% Recovery	Mean % Recovery ± SD	%RSD
1	80%	80	80	79.84	99.80	99.94 ± 0.29	0.29
2	80%	80	80	80.12	100.15		
3	80%	80	80	79.91	99.89		
4	100%	100	100	99.87	99.87	100.02 ± 0.21	0.21
5	100%	100	100	100.24	100.24		
6	100%	100	100	99.94	99.94		
7	120%	120	120	119.76	99.80	100.01 ± 0.24	0.24
8	120%	120	120	120.31	100.26		
9	120%	120	120	119.97	99.98		
Overall						**99.99 ± 0.25**	**0.25**

Precision

Tables 15 and 16 give summary results for the intra-daily and inter-daily precision. The mean peak area for intra-day analysis was 624417 ± 473 mAU·s (RSD = 0.08%) and for inter-day analysis (three consecutive days) was 624411 ± 498 mAU·s (RSD = 0.08%). Retention time %RSD values were all less than 0.30% for all days. Excellent method precision was demonstrated with all values well under ICH Q2(R2) acceptance criterion of ≤ 2.0%.

Table 15: Intra-day Precision (Repeatability) of Finerenone.

Injection No.	Peak Area (mAU·s)	Retention Time (min)
1	624563	3.12
2	623841	3.11
3	625124	3.13
4	624287	3.12
5	623954	3.11
6	624731	3.12
Mean ± SD	**624417 ± 473**	**3.12 ± 0.007**
%RSD	**0.08%**	**0.23%**

Table 16: Inter-day Precision (Intermediate Precision) of Finerenone.

Day	Mean Peak Area ± SD (mAU·s)	%RSD (Peak Area)	Mean Rt ± SD (min)	%RSD (Rt)
Day 1	624417 ± 473	0.08	3.12 ± 0.007	0.23
Day 2	623986 ± 512	0.08	3.11 ± 0.009	0.29
Day 3	624831 ± 489	0.08	3.12 ± 0.008	0.26
Overall Mean ± SD	**624411 ± 498**	**0.08**	**3.12 ± 0.008**	**0.26**
Acceptance Criteria		**≤ 2.0%**		**≤ 2.0%**

Robustness

Robustness was considered by deliberate variation of ethanol proportion, flow rate and buffer pH and the results are presented in Table 17. Negligible change in retention time, tailing factor, theoretical plates and peak area %RSD were seen when ±2% ethanol, ±0.1 mL/min flow rate and ±0.1 pH unit were tried, and %RSD values were all below 0.15%. The optimized method was shown to be robust as it was found to be System suitable under all nine varied conditions within the limits of acceptance.

Table 17: Robustness of Optimized RP-HPLC Method for Finerenone.

Sr. No.	Parameter Varied	Condition	Rt (min)	Tailing Factor	Theoretical Plates (N)	%RSD Peak Area	Inference
1	Ethanol proportion	38% (−2%)	3.38 ± 0.01	1.22 ± 0.02	6712 ± 24	0.14	Robust
2	Ethanol proportion	40% (Optimized)	3.12 ± 0.01	1.18 ± 0.01	6842 ± 19	0.08	Robust
3	Ethanol proportion	42% (+2%)	2.94 ± 0.02	1.21 ± 0.02	6634 ± 31	0.12	Robust
4	Flow rate	0.9 mL/min (−0.1)	3.42 ± 0.01	1.20 ± 0.02	6748 ± 22	0.11	Robust
5	Flow rate	1.0 mL/min (Optimized)	3.12 ± 0.01	1.18 ± 0.01	6842 ± 19	0.08	Robust
6	Flow rate	1.1 mL/min (+0.1)	2.88 ± 0.02	1.21 ± 0.02	6581 ± 28	0.13	Robust
7	Buffer pH	pH 3.4 (−0.1)	3.18 ± 0.01	1.20 ± 0.01	6798 ± 21	0.10	Robust
8	Buffer pH	pH 3.5 (Optimized)	3.12 ± 0.01	1.18 ± 0.01	6842 ± 19	0.08	Robust
9	Buffer pH	pH 3.6 (+0.1)	3.06 ± 0.02	1.22 ± 0.02	6712 ± 26	0.11	Robust

Solution Stability

The solution stability data for finerenone standard solution and sample solution are provided for 24 hours at room temperature and shown in Table 18. Peak values did not change greatly from the initial levels with a maximum percentage change of 0.14% at 24 hrs. The %RSD values were found to be consistently at 0.05% between both the standard and sample solutions, which was within the

acceptable limit of ≤ 2.0%, thus indicating good bench-top stability of the prepared solutions for the period of routine analysis.

Table 18: Solution Stability of Finerenone Standard and Sample Solutions at Room Temperature.

Sr. No.	Time Point	Mean Peak Area — Standard (mAU·s)	%RSD	Mean Peak Area — Sample (mAU·s)	%RSD	% Change from Initial	Inference
1	0 hr (Initial)	624417 ± 312	0.05	623984 ± 287	0.05	—	Stable
2	6 hr	624182 ± 341	0.05	623741 ± 312	0.05	0.04	Stable
3	12 hr	623876 ± 298	0.05	623512 ± 342	0.05	0.09	Stable
4	24 hr	623541 ± 321	0.05	623187 ± 298	0.05	0.14	Stable
Acceptance Criteria			**≤ 2.0%**		**≤ 2.0%**	**≤ 2.0%**	

Filter Compatibility

The results of the filter compatibility tests are displayed in Table 19. The % recovery of finerenone by nylon membrane filter with 0.45 µm pore size was 99.93% with %RSD of 0.05, both of which met the criteria of ≥ 98.0% and ≤ 2.0% respectively. The results verified that there was no adsorption of the drugs on the nylon membrane filter, as well as establishing it for regular filtration of the finerenone solutions prior to their injection into the chromatograph.

Table 19: Filter Compatibility Study of 0.45 µm Nylon Membrane Filter for Finerenone.

Sr. No.	Solution Type	Mean Peak Area (mAU·s)	% Recovery	%RSD	Acceptance Criteria
1	Unfiltered (Centrifuged Reference)	624417 ± 312	100.00	0.05	—
2	Filtered through 0.45 µm Nylon membrane	623984 ± 298	99.93	0.05	98.0–102.0%

Application to Pharmaceutical Formulation

The validated RP-HPLC method was used for the quantitative determination of finerenone in Kerendia® 10 mg tablets successfully and results are summarized in Table 20. The mean value of the percent label claim was 99.97 ± 0.21% with a %RSD of 0.21%, well within the acceptable range of 98.0 – 102.0%. SelGre was confirmed by conducting pharmaceutical formulation analysis in a chromatogram free of interference from tablet excipients showing only a single sharp peak, having a retention time of 3.12 min.

Table 20: Assay of Finerenone in Pharmaceutical Formulation. (Kerendia® 10 mg Tablets)

Sr. No.	Label Claim (mg/tablet)	Amount Found (mg/tablet)	% Label Claim	Mean % Label Claim ± SD	%RSD	Acceptance Criteria
1	10	9.98	99.80	99.97 ± 0.21	0.21	98.0–102.0%
2	10	10.02	100.20			
3	10	9.99	99.90			

DISCUSSION

In the present study, Analytical Quality by Design approach was successfully applied to develop and validate a stability indicating RP-HPLC method, which was developed to quantify finerenone in pure and bulk drug, and demonstrated better chromatographic performance compared to the reported conventional methods. Physicochemical characterization of the bulk drug determined that its melting point was 195.67 ± 0.47°C which falls within the standard reported range (Table 3), and its solubility properties were found to be practical insolubility in water (0.024 mg/mL) and sufficient solubility in HPLC-grade ethanol (68.30 mg/mL), justifying a choice of ethanol as both the stock preparation solvent and the green organic mobile phase modifier (Table 4). The switch from traditional acetonitrile and methanol to ethanol is consistent with the increased awareness and focus on green alternatives for pharmaceutical analysis worldwide, as described by Boussès et al., who have indicated that ethanol is significantly more widely used than other organic solvents for pharmaceutical RP-HPLC methods [33]. The combination of ethanol and 10 mM ammonium formate buffer, pH 3.5, also insured compatibility with mass spectrometry, which is now a requirement for many instruments used in the modern pharmaceutical laboratory. Whereas non-volatile inorganic phosphate buffers are generally used, volatile ammonium salts, like ammonium formate, are becoming the preferred buffers for use with LC-MS [34]. The Full Factorial Design performed with 2 level points in each method parameter (3^2 full factorial), provided the systematic identification of ethanol proportion and flow rate as critical method parameters, and the polynomial models obtained for both the retention time of the main peak (Y_1; linear, Adjusted R2 = 0.9603) and the tailing factor of the main peak (Y_2; quadratic, Adjusted R2 = 0.9170), were well statistically significant (Tables 6–7): with the optimum point of design (Table 4 for Run 5, 40% ethanol, 1.0 mL/min) providing the best chromatographic conditions (Retention time (Rt) = 3.12 min, Tailing factor (tf) = 1.18, and N = Number of theoretical plates = 6842). This method goes beyond the basic capability of a one factor at a time approach that does not consider interactions and is officially recognized via ICH Q14 and supported by reports from Analytical Chemistry that combine AQbD with lifecycle management [35]. The present method is competitive to the existing finerenone RP-HPLC literature due to its various unique features. Illendula et al. used methanol phosphoric buffer 35:65 v/v for isocratic retention time of 3.006 min, with detection at 235 nm and linearity of 6-14 µg/mL; Mohammed et al. used acetonitrile: water (50:50 v/v) with detection at 252 nm for isocratic retention time measurement of 4-6 min [36]. Notably,

Murugesan et al. and others who use the same mobile phase systems in assays with phosphate or acetonitrile buffers published non-MS-compatible methods with only limited use in the regulatory arena as a basis [37]. Through forced degradation studies, the stability indicating capability of finerenone was unambiguously confirmed (Table 11): The alkaline hydrolysis showed that Finerenone is the most susceptible degradation method (15.68% degradation, three peaks) followed by acid hydrolysis (18.26% degradation, two peaks) and oxidative stress (21.46% degradation, three peaks) in which the degradation products are completely separated from the main peak (resolution ≥ 2.0) as reported by Saad et al in Scientific Reports (18.26% degradation in acid, two peaks, 18.26% in oxidative stress, two peaks, 21.46% in alkaline hydrolysis, three peaks) [38]. Comparatively less loss of the drug was detected under the thermal and photolytic conditions (7.82% and 11.54% respectively; Table 11) in line with earlier observations made by Murugesan et al. which suggested that the dihydropyridine-naphthyridine scaffold was selectively vulnerable to hydrolysists under alkaline and oxidative microenvironments [39].

The optimized method was validated for all the parameters investigated by comprehensive ICH Q2(R2) validation demonstrating fitness-for-purpose of the optimized method. The %RSD of peak area was very stable (i.e., 0.08%) and the tailing factor and the theoretical plate count were within USP and ICH "good" criteria: 1.18 ± 0.007; 6845 ± 19.4. Specificity and peak purity confirmation (Table 10) showed a purity angle of 0.142 and 0.156; these were both below the established purity angles of 0.387 and 0.412, respectively, indicating spectral homogeneity of spectrometric quality of finerenone peak in all the solution types. As can be seen in Table 12, the device is linear over the range of 10-120 µg/mL and the regression equation y = 6241.8x + 312.4 with the coefficient of determination $r^2 = 0.9998$, is quite linear in comparison to the working range of 6-14 µg/mL and 10-50 µg/mL demonstrated by Illendula et al. and Doserge et al. respectively [40], The values for detection limit, 0.026 µg/mL and quantification limit, 0.078 µg/mL (Table 13), proven experimentally by the signal-to-noise ratio values, showed a significant improvement in the detection capability of these analytes when compared with the LOD and LOQ for methods listed as isocratic with acetonitrile: methanol, 1.1 µg/mL and 3.2 µg/mL respectively. Accuracy at the 80%, 100%, and 120% levels yielded mean recoveries of 99.94%, 100.02%, and 100.01% respectively (Table 14), and both intra-day and inter-day precision exhibited %RSD below 0.30% (Tables 15–16), well surpassing the ICH Q2(R2) criterion of ≤ 2.0%. Deliberate perturbations of ethanol proportion ±2%, buffer pH ±0.1 unit and flow rate ±0.1 mL/min were introduced into the Chromatographic System Suitability tests (Table 17) which showed an operational robustness of the method with peak area %RSD below 0.15% for all perturbations, a result that confirms the MODR validation in multi-analyte systems undertaken by Gamal et al. within AQbD design [41]. The method was practically suitable for use in the routine laboratory, demonstrated by solution stability for 24 hours (Table 18) and for passage through 0.45 µm nylon membrane (Table 19, 99.93% recovery). The mean label claim found in the formulation assay for Kerendia® 10 mg tablets was 99.97 ± 0.21% (Table 20), and this was in good agreement

with the results obtained by Jilla et al. and Mohammed et al., who also obtained good drug content in their conventional non-QbD RP-HPLC assay of the same formulation [42]. Together, the current method constitutes the first regulatory-compliant AQbD approach for the quantification of finerenone in ethanol with minimal changes to the RP-HPLC methodology, based on ICH Q14 guidelines, as well as environmental-friendly solvents and analytically fresh and better sensitivity and linearity than all previously published methods.

CONCLUSION

In conclusion, a successful stability-indicating RP-HPLC method for the quantitative determination of finerenone, developed using the Analytical Quality by Design system fulfilled with ICH Q14 and ICH Q 2(R2) guidelines in its pure and bulk drug form was successfully developed and validated. The full-fractional design 3^2 optimization resulted in the mobile phase system ethanol–ammonium formate, which showed excellent chromatographic separation in an environmentally friendly mode and fulfilled all the validation parameters in compliance with the regulatory criteria of specificity, linearity, accuracy, precision, sensitivity, robustness, and stability. This assay method is clinically relevant to guaranteeing the drug quality, reliable pharmacokinetic monitoring, and therapeutic drug management of Kerendia® tablets because the method has shown to be confirmed and stable.Kerendia® tablets' stability-indicating capability and precise formulation assay reinforces the relevance of this assay method in order to ensure drug quality, support pharmacokinetic monitoring, and to facilitate TDM in a cardiorenal patient. Future studies including in vivo pharmacokinetic studies and bioanalytical expansion to other plasma matrices would further validate the method application in the context of finerenone clinical research and regulatory submissions.

ABBREVIATIONS

AQbD: Analytical Quality by Design; ATP: Analytical Target Profile; CKD: Chronic Kidney Disease; CV: Cardiovascular; DoE: Design of Experiments; FDA: Food and Drug Administration; HPLC: High-Performance Liquid Chromatography; ICH: International Council for Harmonisation; LOD: Limit of Detection; LOQ: Limit of Quantification; MRA: Mineralocorticoid Receptor Antagonist; MODR: Method Operable Design Region; MS: Mass Spectrometry; ODS: Octadecylsilane; RP: Reversed-Phase; RSD: Relative Standard Deviation; SD: Standard Deviation; T2DM: Type 2 Diabetes Mellitus; UV: Ultraviolet; USP: United States Pharmacopoeia.

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