# Development of a Validated RP-HPLC Method Using Full Factorial Design for the Analysis of Ramipril

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**(Received: March 21, 2024; Accepted: May 22, 2024; Published (web): June 13, 2024)**

**ABSTRACT:** The primary objective of this study is to develop and optimize a simple, novel, reproducible, and efficient RP-HPLC method using quality by design (QbD) approach for the routine analysis of ramipril. The chromatographic separation was carried out by C18 column with an isocratic elution of a mobile phase of 65:35 (%v/v) acetonitrile: water at a flow rate of 0.9 mL/min. The detection was done at a wavelength of 210 nm using a photo-diode array plus (PDA+) detector. A $3^2$ full-factorial design was employed for the development of analytical method using Design Expert® software in which the mobile phase composition and flow rate were taken as independent variables and the retention time (RT), tailing factor (TF) and theoretical plate count (TP) were chosen as responses of the study. Statistically significant models were obtained for the development of the method ($p$<0.05). The empirical responses perfectly fitted to that of predicted, with an error within the tolerance of ± 2%. This indicates that the model efficiently identified the optimum levels of independent variables, i.e. the composition of mobile phase and its flow rate, to get the desirable responses. The validation of the developed method followed the ICH Q2 (R1) guidelines, demonstrating that the method is robust and well-suited for the routine analysis of ramipril in active pharmaceutical ingredients and in drug products.

**Key words:** Ramipril, analytical method, RP-HPLC, full-factorial design, quality by design (QbD), ICH guidelines.

## INTRODUCTION

Hypertension is a serious and potentially life-threatening medical condition which arises from the persistent elevation of blood pressure.[1] According to World Health Organization report in 2023, an estimated 1.28 billion adults between the ages of 30 and 79 worldwide have hypertension, with most of them living in low- and middle-income countries.[2] Ramipril, an inhibitor of angiotensin converting enzyme (ACE), acts by inhibiting the conversion of angiotensin I to angiotensin II, resulting in vasodilation, and also contributes to the loss of sodium and water.[3] This dual mechanism helps to lower blood pressure and reduce the workload on the heart, making it an effective treatment for conditions like hypertension and heart failure.[4]

Quality by design (QbD) is a systematic and science-based approach to product and process development that focuses on integrating quality into a product or process from the very beginning rather than relying solely on testing and inspection at the end of a production.[5] In the development of analytical methods, QbD principles entail adopting a systematic and scientific methodology to comprehend and

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Dhaka Univ. J. Pharm. Sci. **23**(1): 93-102, 2024 (June)
DOI: https://doi.org/10.3329/dujps.v23i1.74098

control the method's performance attributes. This approach equips analytical scientists with a profound knowledge of the workings of process parameters.[6] Through the application of QbD, the emphasis shifts from conventional trial-and-error techniques to a more methodical and scientifically grounded approach which contributes to the development of reliable and resilient analytical method.[7]

Analytical method and product development go hand in hand throughout the life cycle of any pharmaceutical product.[5] Currently, there is no specific, economical and dedicated RP-HPLC method for the determination of ramipril in dosage forms using the QbD approach. After thoroughly reviewing literature, it was apparent that majority of the methods entail expensive, laborious, and intricate procedures.[8-11] The method proposed by Rao *et al.* for analysis of ramipril involves troublesome mobile phase preparation.[8] Sunil *et al.* demonstrated a HPLC method where the retention time for ramipril was 10.8 min, which refers that the method takes more time and is solvent consuming.[9] Lakshmi *et al.* proposed a stability indicating HPLC method having retention time of ramipril at 1.91 min which could lead to poor separation of analytes from sample.[10] Hanysov *et al.* studied the stability of ramipril in the solvents of different pH using HPLC where costly reagents were used.[11]

Therefore, the goal of this study is to develop a simple and efficient RP-HPLC method for routine analysis of ramipril.

## MATERIALS AND METHODS

**Chemicals and reagents.** Ramipril powder (purity >99.91%; BEC Chemical, India) was gifted by the Healthcare Pharmaceuticals Ltd. (Dhaka, Bangladesh). HPLC grade acetonitrile used for the preparation of the mobile phase in the experiment was purchased from Merck, Germany. The Milli-Q system (Millipore, USA) was employed to purify the nano-purified water used in the experiment.

**Chromatographic conditions.** The RP-HPLC system containing Shimadzu LC-2050 series equipped with an auto-sampler, a column oven, a vacuum degasser and a PDA plus detector linked to computer running LabSolutions software platform, was used for the analysis. The separations were carried out on Inertsil ODS C18 column having 250 mm × 4.6 mm internal diameter with 5 µm particle size. Acetonitrile and nano-purified water at the ratio of 65:35 %v/v were used as mobile phase, with a total run time for 10 min. The injection volume was 10 µL, and the detection was done at a wavelength of 210 nm.

**Preparation of stock and standard solutions.** Accurately weighed 1 mg of ramipril working standard was carefully taken into a 10 mL volumetric flask. This was dissolved in methanol and the final volume was adjusted to 10 mL using the same solvent, sonicated and degassed for 5 min. The final stock concentration was 100 µg/mL, and was further diluted to obtain the desired standard concentrations using the same solvent.

**Preparation of mobile phase.** Nano-purified water was filtered with 0.45 µm filter paper, and then degassed through sonication for 30 min. The HPLC grade acetonitrile was also filtered and degassed in the same way prior to use.

**Development of HPLC method.** A new RP-HPLC method was developed and optimized using a mobile phase containing mixture of acetonitrile and nano-purified water for achieving optimum chromatographic separation. Design Expert® software (version 13) was used to construct a $3^2$ full factorial design to analyze the correlation between independent variables and response. Here, the independent variables were the composition of mobile phase and flow rate, and the responses were retention time (RT) (R1, min), tailing factor (R2) and theoretical plate count (TP) (R3, N). For designing the experiment, three levels (low, medium and high) of each independent variable were chosen (Table 1). The significance of the design was evaluated by analyzing the statistical parameters such as ANOVA method and good fit evaluation. Analysis by response surface methodology was used for the optimization of the method parameters.

**Validation of the method.** The validation of the developed RP-HPLC method for ramipril was carried out in accordance with the ICH Q2 (R1) guidelines for system suitability, specificity, linearity, accuracy, precision, ruggedness and robustness.[12,13]

**System suitability.** The system suitability of the proposed method was determined using values of the peak area, retention time, tailing factor and theoretical plate of six replicate analyses of standard solution (50 µg/mL) of ramipril. The results were assessed as the percent relative standard deviation (% RSD).

**Table 1. Independent variables and their levels.**

| Variables | Name | Unit | Type | Coded value | | | Actual value | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Low | Mid | High | Low | Mid | High |
| A | Acetonitrile | % | Numeric | -1 | 0 | 1 | 55 | 60 | 65 |
| B | Flow rate | ml/min | Numeric | -1 | 0 | 1 | 0.7 | 0.8 | 0.9 |

**Specificity.** This involves analyzing a sample that contains only the analyte of interest (pure ramipril). However, the peak corresponding to the analyte should be well-defined and not affected by any co-eluting component.

**Linearity.** The linearity of the developed RP-HPLC method was measured by analyzing ramipril covering 80% to 120% of the nominal concentration (50 µg/mL). The standard curve was constructed using the concentrations of 10, 20, 30, 40, 45, 50, 55 and 60 µg/mL. Regression analysis was done to assess the linearity of the method.

**Accuracy.** Recovery tests for the drug solution were used to determine the accuracy of the suggested method. Percent recoveries of ramipril were calculated with the concentrations ranging 10-60 µg/mL.

**Precision.** The standard solution of ramipril (50 µg/ml) were analyzed in six replicates on the same day and daily for six times over the course of three days in order to measure the intra-day precision (repeatability) and inter-day precision of the procedure. The results were demonstrated as % RSD.

**Sensitivity.** For sensitivity study, baseline parameter was investigated. Afterward, the highly diluted solutions of ramipril were analyzed. The chromatograms with the signal-to-noise ratio at 3:1 and 10:1 were established as the limit of detection (LOD) and limit of quantification (LOQ) values, respectively.

**Ruggedness.** Six assay sample solutions of nominal standard concentration were examined in two separate laboratories by two different analysts to obtain the reproducibility of the results. The values of percent recovery and the %RSD were used to determine the ruggedness.

**Robustness.** To measure the robustness of the method, the responses of the method to minor variations in flow rate, mobile phase composition and wavelength were assessed. The effect of flow rate was investigated at 0.8 mL/min and 1 mL/min other than 0.9 mL/min. The effect of mobile phase composition was investigated at acetonitrile: water at the ratio of 62.5:37.5 and 67.5:32.5 %v/v instead of 65:35 %v/v. The effect of wavelength was investigated at 205 nm and 215 nm other than 210 nm.

## RESULTS AND DISCUSSION

**Analysis of responses.** A $3^2$ full factorial design was utilized to conduct nine investigational runs for observing the effect of two independent variables, i.e. the percentage of acetonitrile (A) and flow rate (B), on three responses (retention time, tailing factor and theoretical plate) for the development of the RP-HPLC method using Design Expert® software (version 13) (Table 2). All three responses (RT, TF and TP) followed linear mathematical models depicting their correlation with the chosen method parameters (mobile phase composition and flow rate).

The ANOVA results for R1, R2 and R3 responses indicated that the model is statistically significant ($p$ <0.05). For response 1 (retention time), both the model terms, i.e. % of acetonitrile in mobile phase and flow rate of mobile phase, are statistically significant ($p$ <0.05), indicating that they have strong impact on the retention time of ramipril. For responses 2 and 3, however, only the model term B (flow rate of mobile phase) is statistically significant ($p$ <0.05). This indicates that variation in the flow rate of mobile phase causes significant changes in the both tailing factor and theoretical plate count of the method. The predicted $R^2$ values for all responses R1 (0.9061), R2 (0.9243) and R3 (0.6214) were in acceptable agreement with the adjusted $R^2$ values of 0.9416, 0.9502 and 0.7249, respectively, as the difference is less than 0.2 in every case. Adequate precision is used to measure signal-to-noise ratio, and its value greater than 4 is highly desirable. The adequate precision values for all three responses were found to be 21.5687, 19.6608 and 7.8886, indicating an adequate signal. Hence, design space can be navigated by using these models.

**Table 2. Full factorial design ($3^2$) for the development of RP-HPLC method.**

| Runs | Factor A: % of ACN | Factor B: Flow rate (mL/min) | Response 1 RT (min) | Response 2 TF | Response 3 TP |
|---|---|---|---|---|---|
| 1 | 60 | 0.9 | 4.21 | 1.08 | 2602 |
| 2 | 65 | 0.8 | 4.26 | 1.15 | 2331 |
| 3 | 65 | 0.9 | 4.02 | 1.09 | 2511 |
| 4 | 55 | 0.8 | 4.64 | 1.20 | 2134 |
| 5 | 55 | 0.9 | 4.28 | 1.09 | 2511 |
| 6 | 60 | 0.7 | 4.70 | 1.29 | 2040 |
| 7 | 60 | 0.8 | 4.55 | 1.15 | 2066 |
| 8 | 55 | 0.7 | 4.81 | 1.27 | 2103 |
| 9 | 65 | 0.7 | 4.66 | 1.25 | 2146 |

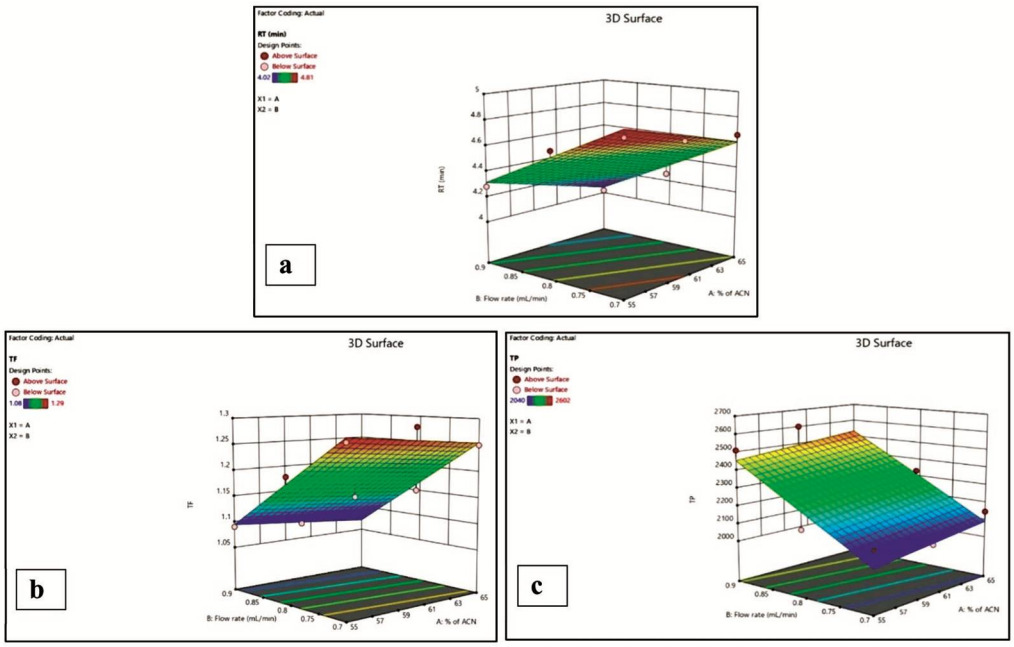


Figure 1. Interaction between the RP-HPLC method parameters (mobile phase composition and flow rate) and responses (RT, TF and TP). The 3D response surface plots showing the impact of variables for proposed RP-HPLC method, i.e. % of acetonitrile in mobile phase and flow rate of mobile phase, on (a) response 1 (RT), (b) response 2 (TF) and (c) response 3 (TP).

By analyzing 3D response surface plot and model equation, it was found that both model terms A and B (i.e. % of acetonitrile in mobile phase and flow rate of mobile phase) are negatively correlated with the retention time of ramipril (R1) (Figure 1a and Table 3). This implies that increasing the levels of both method parameters decreases the retention time of ramipril. The statistical significant model term B (i.e. flow rate of mobile phase) negatively affects the tailing factor of the method (Figure 1b and Table 3). This means if the flow rate of the mobile phase is increased the tailing factor of the ramipril's peak in the chromatogram is decreased. In contrast, the flow rate of the mobile phase is positively correlated with the theoretical plate count of the method, indicating that if flow rate is increased the theoretical plate count is also increased accordingly (Figure 1c and Table 3).

**Table 3. ANOVA and regression equation of responses.**

| ANOVA for the responses | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | R1 | | | R2 | | | R3 | | |
| Source | SS* | F | P | SS | F | P | SS | F | P |
| Model | 0.5633 | 65.48 | < 0.0001 | 0.0512 | 77.28 | < 0.0001 | 3.066E+05 | 11.54 | 0.0088 |
| A-% of ACN | 0.1040 | 24.18 | < 0.0027 | 0.0008 | 2.46 | 0.1676 | 9600.00 | 0.7225 | 0.4279 |
| B-Flow rate | 0.4593 | 106.78 | < 0.0001 | 0.0504 | 152.09 | < 0.0001 | 2.970E+05 | 22.35 | 0.0032 |
| Residual | 0.0258 | | | 0.0020 | | | 79724.72 | | |
| Cor Total | 0.5891 | | | 0.0532 | | | 3.864E+05 | | |

| Fit statistics | | | | Regression equation |
|---|---|---|---|---|
| Source | R1 | R2 | R3 | R1 = +8.25222-0.026663A-2.76667B |
| Std. Dev. | 0.0656 | 0.0182 | 115.27 | |
| Mean | 4.46 | 1.17 | 2271.56 | R2 = +2.04778-0.002333A-0.916667B |
| C.V. % | 1.47 | 1.55 | 5.07 | |
| $R^2$ | 0.9562 | 0.9626 | 0.7937 | R3 = +11.55556+8.0A+2225.0B |
| Adjusted $R^2$ | 0.9416 | 0.9502 | 0.7249 | |
| Predicted $R^2$ | 0.9061 | 0.9243 | 0.6214 | |
| Adeq Precision | 21.5687 | 19.6608 | 7.8886 | |

*sum of square.

**Optimization of the method.** Out of nine solutions provided by the Design Expert® software, the optimized method was taken as the method having the highest desirability level which involved the use of variable A (% of acetonitrile in mobile phase) as 65% and variable B (flow rate of mobile phase) as 0.9 mL/min, with the desirability of 0.991. The criteria used for the optimization of the method were given in the table 4. Experimental values of the responses were in agreement to that of the predicted responses, as the tolerance is set for ±2% (Table 5). The chromatogram of ramipril obtained from the optimized method is presented in figure 2.

**Table 4. Optimization criteria for responses.**

| Responses | Criteria |
|---|---|
| Response 1 (Retention time, RT) | Minimize |
| Response 2 (Tailing factor, TF) | Minimize |
| Response 3 (Theoretical plate count, TP) | Maximize |

**Validation of the RP-HPLC method.**

**System suitability.** The values of the peak area, retention time, tailing factor and theoretical plate of six replicate analyses of working standard solution of ramipril were within the acceptable ranges, indicating that the method is suitable and generates reproducible results (Figure 3 and Table 6).

**Table 5. Optimized method and predicted errors of responses.**

| Method | Percentage of acetonitrile | Flow rate (mL/min) | Response 1 (RT) | Response 2 (TF) | Response 3 (TP) |
|---|---|---|---|---|---|
| Predicted values | 65 | 0.9 | 4.05 | 1.07 | 2534.05 |
| Experimental values | 65 | 0.9 | 4.13 | 1.09 | 2555.10 |
| *Predicted errors (%) | | | 1.94 | 1.83 | 0.82 |

*Predicted errors (%) = [(Experimental value – Predicted value)/Experimental value] × 100%; Tolerance: ± 2%

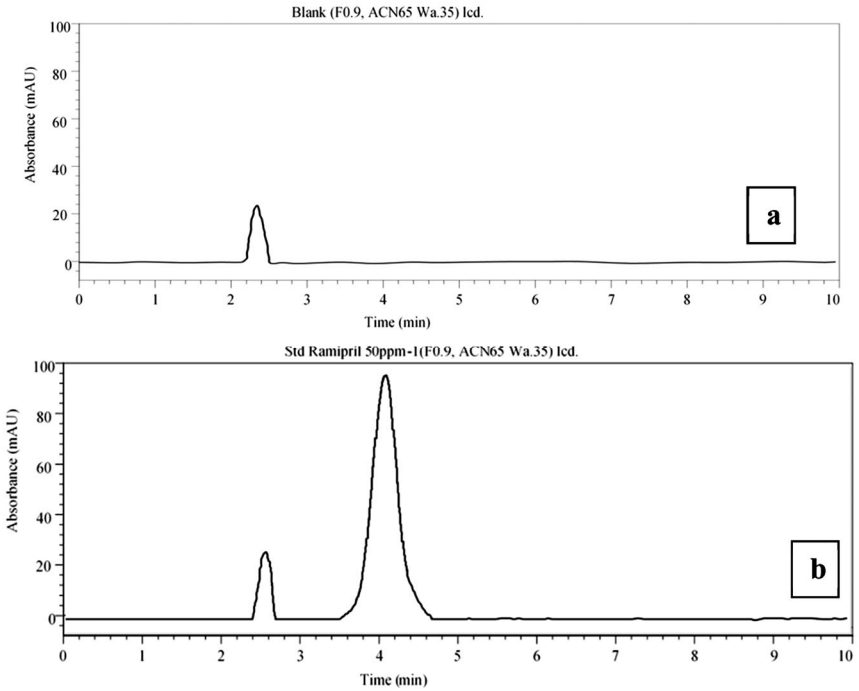


Figure 2. Chromatogram for the optimized RP-HPLC method of ramipril. (a) Blank chromatogram and (b) chromatogram of ramipril standard solution (concentration: 50 µg/mL).

**Specificity.** The chromatogram for ramipril showed an excellent resolution with blank showing no peak during the retention time, indicating that the method is specific (Figure 4).

**Linearity.** The correlation coefficient ($R^2$) value for linearity study was found to be 0.9998 with the concentration range of 10-60 µg/mL, referring that the method shows linear response with the concentration variation (Figure 5).

**Accuracy.** The recovery values of ramipril ranged from 99.98±0.009% to 101.003±0.024% (Mean±% RSD), suggesting a good accuracy of the method (Table 6).

**Precision.** The intra-day and inter-day precision studies indicated that the method is highly precise, as there was no significant difference between the assay results of solutions within a day or between days (Table 6).

**Sensitivity.** The limit of detection (LOD) and limit of quantification (LOQ) of ramipril were found to be 0.8 µg/mL and 2.5 µg/mL, respectively (Figure 6). This indicates that the method is highly sensitive, and could be used to trace and analyze a very low quantity of ramipril.

**Ruggedness.** The %RSD of the ruggedness analysis of the proposed method from analyst 1 and

analyst 2 were found to be 0.015% to 0.47%, respectively, indicating that the method is rugged (Table 6).

**Robustness.** The robustness of the method as assessed by changing the several method parameters (flow rate and composition of mobile phase and wavelength) showed that the percent recovery values of the standard solution (50 µg/mL) were within the acceptable ranges (%RSD <2%) (Table 6).

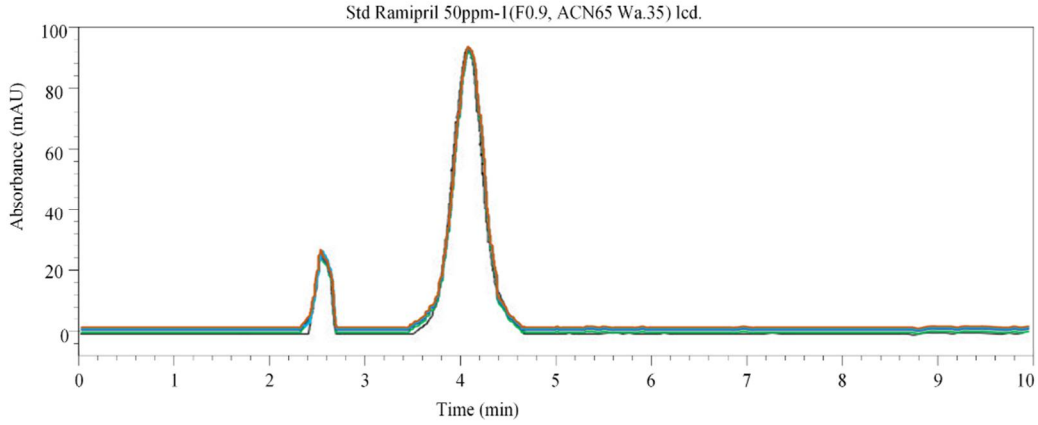


Figure 3. System suitability study of the RP-HPLC method for ramipril. An overlay plot of six replicate analyses of working standard solution of ramipril (concentration: 50 µg/mL).

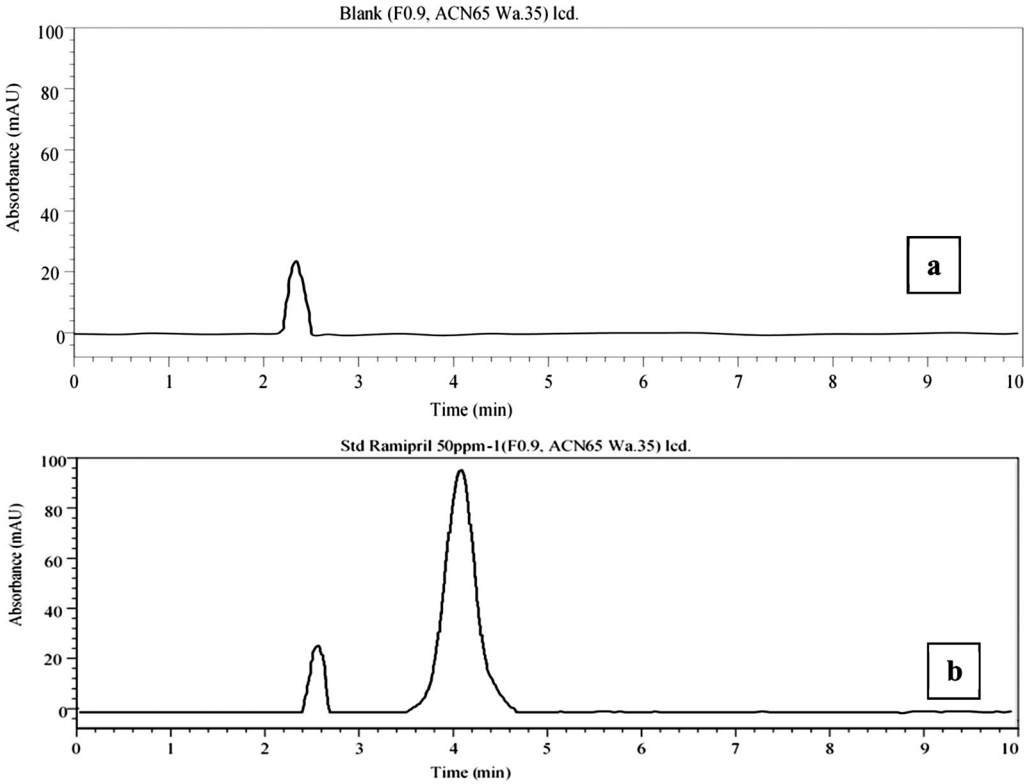


Figure 4. Specificity study of the RP-HPLC method for ramipril. Chromatograms for (a) blank sample and (b) standard solution of ramipril (concentration: 50 µg/mL).

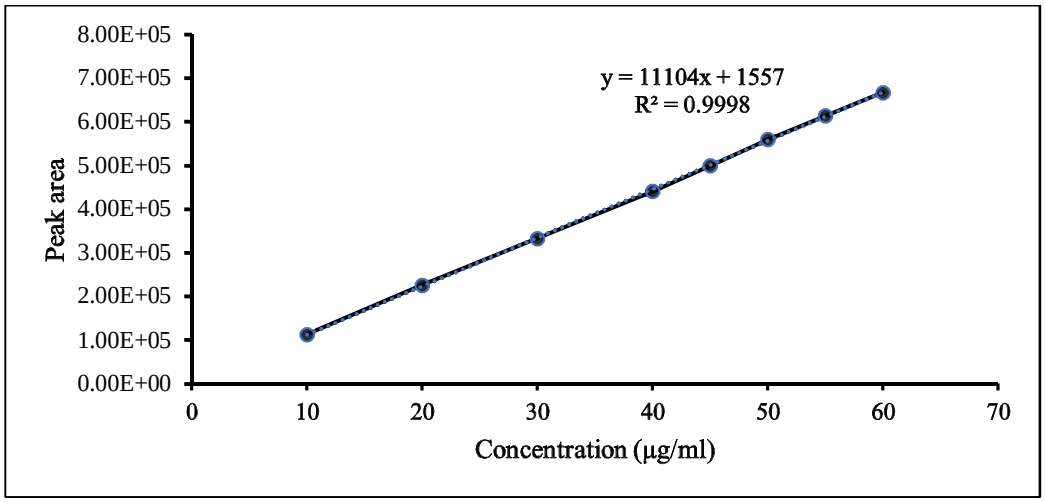


Figure 5. Standard curve for ramipril (concentration range: 10-60 µg/mL). The standard curve is constructed by analyzing different concentration solutions of ramipril (10, 20, 30, 40, 45, 50, 55 and 60 µg/mL) with the RP-HPLC method. The peak areas are plotted against the corresponding concentrations using MS Excel.

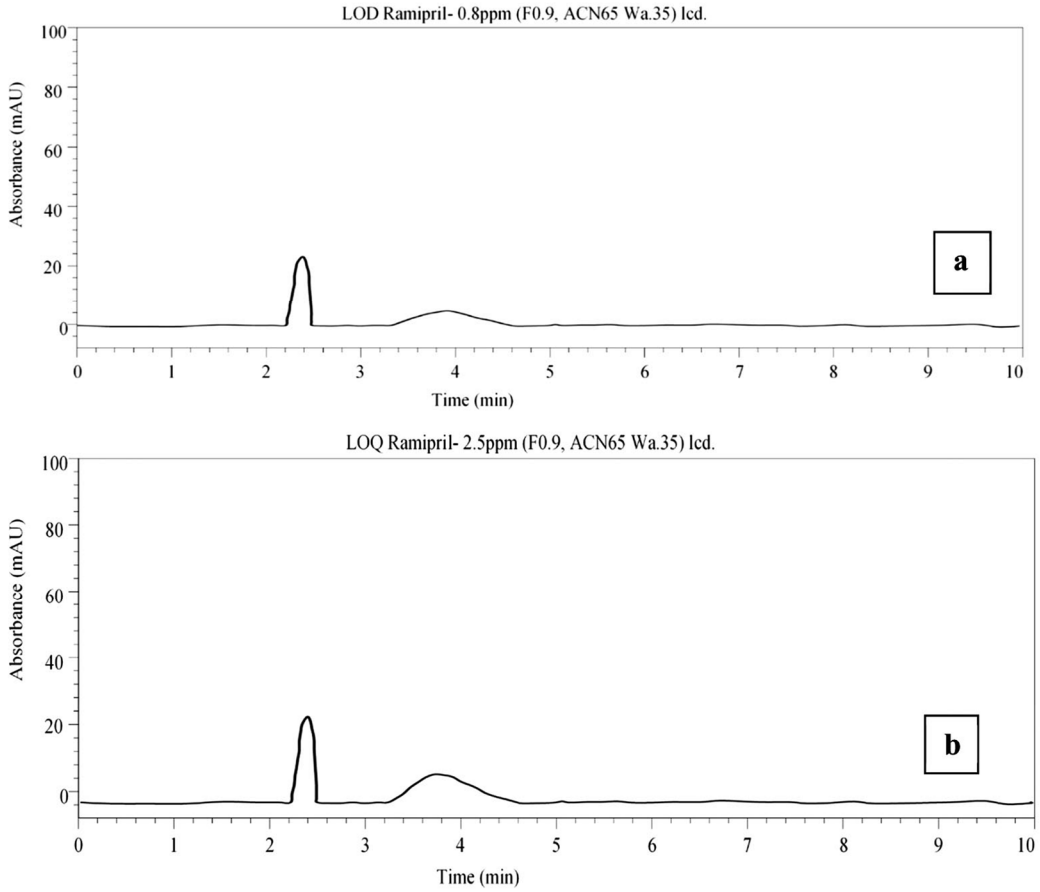


Figure 6. Sensitivity study of the RP-HPLC method for ramipril. Chromatograms for (a) limit of detection (LOD) at 0.8 µg/mL and (b) limit of quantitation (LOQ) at 2.5 µg/mL.

**Table 6. Validation of the RP-HPLC method for ramipril.**

| Type of study | Values (Mean±%RSD) | Acceptable limits | Type of study | Amount added (µg/mL) | Mean % recovery | %RSD |
|---|---|---|---|---|---|---|
| System suitability | | | Accuracy | | | |
| Peak area | 565124.4±0.98% | %RSD ≤ 2 | | 10 | 100.020 | 0.031 |
| Tailing factor | 1.09± 1.58% | ≤ 1.5 | | 20 | 100.001 | 0.016 |
| Theoretical plate | 2,567.5± 0.79% | >2000 | | 30 | 100.008 | 0.007 |
| Retention time | 4.065± 0.46% | %RSD ≤ 0.5 | | 40 | 99.980 | 0.014 |
| | | | | 45 | 99.980 | 0.009 |
| | | | | 50 | 100.007 | 0.016 |
| | | | | 55 | 99.990 | 0.010 |
| | | | | 60 | 101.003 | 0.024 |

| Type of study | Spike level (%) | Type of precision | | Mean % recovery | %RSD |
|---|---|---|---|---|---|
| Precision | 50 | Intra-day | | 100.005 | 0.012 |
| | | Inter-day | Day-1 | 100.008 | 0.017 |
| | | | Day-2 | 100.015 | 0.014 |
| | | | Day-3 | 100.010 | 0.011 |

| Type of study | Type of ruggedness | Mean % recovery | %RSD |
|---|---|---|---|
| Ruggedness | Analyst-1 | 100.011 | 0.015 |
| | Analyst-2 | 99.710 | 0.471 |

| Type of study | Parameter | Variation | Amount added (µg/mL) | Mean % recovery | %RSD |
|---|---|---|---|---|---|
| Robustness | Mobile phase Flow rate (mL/min) | 0.8 | 50 | 100.023 | 0.003 |
| | | 0.9 | 50 | 100.007 | 0.006 |
| | | 1.0 | 50 | 99.710 | 0.530 |
| | Mobile phase composition (Acetonitrile: water) | 62.5:37.5 | 50 | 99.710 | 0.940 |
| | | 65:35 | 50 | 99.990 | 0.020 |
| | | 67.5:32.5 | 50 | 100.018 | 0.018 |
| | Wavelength | 205 nm | 50 | 99.990 | 0.018 |
| | | 210 nm | 50 | 100.010 | 0.023 |
| | | 215 nm | 50 | 100.012 | 0.018 |

## CONCLUSION

A novel, rapid and simple RP-HPLC method is developed for the analysis of ramipril using QbD approach. The optimized method is validated in accordance to the ICH Q2 (R1) guidelines and found to be selective, specific, robust, linear, accurate and precise, with lower detection and quantitation limits. Thus, the proposed method could be used for the quantitation of ramipril in bulk drug and in formulation during the manufacture of pharmaceutical products to ensure their safety and efficacy.

## ACKNOWLEDGEMENT

The authors are thankful to Healthcare Pharmaceuticals Ltd. (Dhaka, Bangladesh) for providing the necessary raw materials for the research work.

## AUTHORS CONTRIBUTIONS

The conceptualization, supervision, manuscript writing and editing were done by UK. The investigation, methodology, statistical analysis data curation, original manuscript writing was done by JH and DKS. The data curation and manuscript

preparation (review and editing) were performed by SCD, KSA and HH.

## CONFLICT OF INTEREST

The authors declare 'no conflict of interest'.

## FUNDING INFORMATION

The research work has been carried out by the R&D project grant for the fiscal year 2022-2023, Ministry of Science and Technology, Government of Bangladesh (Group: Biology, Medical Science and Nutrition Science; Serial No. 51; Registration No. 70; GO No. 39.00.0000.012.20.011.22.56, dated 03 April 2023).

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