

# Rapid Simultaneous Quantitative Analysis of Hypoglycemic agents by RP HPLC: Development, Validation and Application to Medicine

Mahesh Attimarad<sup>1,\*</sup>, Katharigatta Narayanaswamy Venugopala<sup>1,2</sup>, Mohammed Munirul Islam<sup>3</sup>, Sheeba Shafi<sup>4</sup>, Abdulrahman Ibrahim Altaysan<sup>1</sup>

<sup>1</sup>Department of Pharmaceutical Sciences, College of Clinical Pharmacy, King Faisal University, Al Ahsa, SAUDI ARABIA.

<sup>2</sup>Department of Biotechnology and Food Science, Faculty of Applied Sciences, Durban University of Technology, Durban, SOUTH AFRICA.

<sup>3</sup>Department of Biomedical Sciences, College of Clinical Pharmacy, King Faisal University, Al Ahsa, SAUDI ARABIA.

<sup>4</sup>Department of Nursing, College of Applied Medical Sciences, King Faisal University, Al Ahsa, SAUDI ARABIA.

## ABSTRACT

**Background:** For the treatment of diabetes mellitus type 2, a new formulation containing vildagliptin and remogliflozin was developed. A simple and rapid RP-HPLC method employing linagliptin as an internal standard was developed for quality control of this medicine. **Methodology:** Formulation analytes, including IS, were separated on a Zorbax C<sub>18</sub> column with isocratic elution of acetonitrile and phosphate buffer (pH 5) 55:45 v/v at a flow rate of 1.2 mL/min. The experiment was carried out at room temperature and monitored at a wavelength of 210 nm. The approach was also validated in accordance with the ICH Q2 requirements. **Results:** The optimized HPLC approach revealed a satisfactory linearity in the concentration ranges of 10-60 µg/mL and 10-100 µg/mL for VIL and REM respectively, with good regression coefficient ( $R^2 \geq 0.998$ ). The average accuracy for VL and REM was 99.57 percent and 100.59 percent, respectively, with a low percentage relative error. The method's precision was proven by the low percentage relative standard deviation. Furthermore, a robustness assessment employing a Pareto chart generated using a three-level factor interaction study, a multivariate technique, demonstrated that minor changes in individual experimental conditions had no effect on the test results. Finally, the optimized HPLC method was effectively used to assess VIL and Rem from formulation simultaneously. **Conclusion:** The findings of an assay comparing a simple and rapid isocratic RP-HPLC method devised for the simultaneous quantification of VIL and REM to a previously published approach revealed no significant differences in the assay results. As a result, it might be utilized in any analytical laboratory for quality control of this formulation.

**Key words:** Vildagliptin, remogliflozin, HPLC method, Validation, Formulation, Robustness, Multivariate.

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Correspondence:

Dr. Mahesh V Attimarad

Department of Biomedical Sciences, College of Clinical Pharmacy, King Faisal University, Al Ahsa, SAUDI ARABIA.

E-mail: mattimarad@kfu.edu.sa

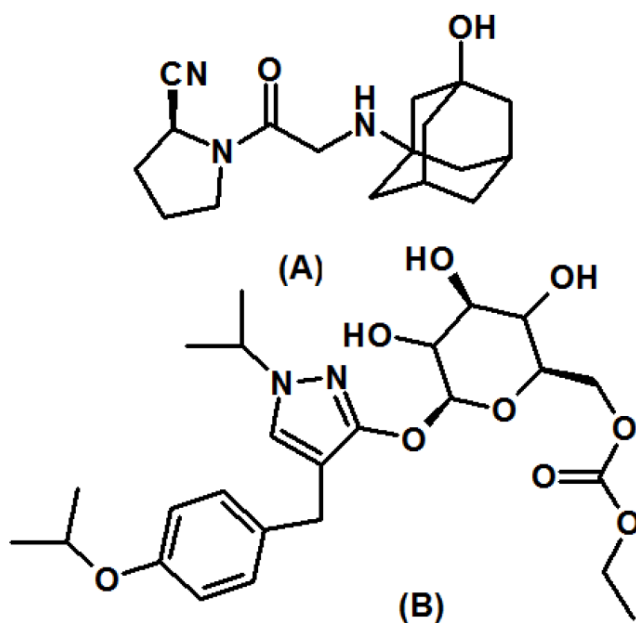
## INTRODUCTION

Diabetes mellitus type-2 (DMT2) is metabolic disorder characterized by high blood sugar levels due to insufficient insulin synthesis or body cells that do not absorb glucose energy conversion. DMT-2 is particularly frequent in the elderly; one in every four adults aged 65 and up is diabetic.<sup>1-3</sup> However, DMT-2 affects one out of every ten people of all ages. In diabetic individuals, the number of

COVID-19 patients admitted to hospitals and deaths is likewise high.<sup>4</sup> Maintaining a normal blood glucose level is critical for avoiding problems and organ failure such as, kidneys, heart, neurological system, and eyes. To maintain a normal blood glucose level 2, pharmacological treatment with oral diabetes drugs, as well as physical activity, healthy eating, and reduced mental stress, is required. To attain a low HbA<sub>1c</sub> level, several



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**Figure 1: Chemical structures of vildagliptin (A) and remogliflozin etabonate (B).**

oral antidiabetic medicines are being researched, including dipeptidyl peptidase-4 (DPP-4)<sup>5-7</sup> and sodium glucose cotransporter-2 (SGLT-2) inhibitors.<sup>8</sup> Vildagliptin (VIL, Figure 1A) works by blocking the DPP-4 enzyme, which is responsible for the breakdown of GLP-1 and glucose-dependent insulinotropic polypeptide, resulting in increased insulin secretion and glucagon secretion. Remogliflozin etabonate (REM, Figure 1B) works by blocking the SGLT-2 enzyme, which causes a rise in glucose excretion in the urine. Furthermore, SGLT-2 has the added advantage of lowering body weight and blood pressure.<sup>9-11</sup> The combination of VIL and REM improved glycemic control and provided additional benefits.<sup>12</sup>

VIL analysis from pharmaceutical formulations and biological samples was reported in the literature utilizing UV-Vis spectrophotometry,<sup>13</sup> spectrofluorometric,<sup>13,14</sup> capillary electrophoresis,<sup>15,16</sup> RP-HPLC,<sup>17,18</sup> HPTLC<sup>19</sup> and LC-MS.<sup>20</sup> Quantification of REM from formulations was reported using spectrophotometry,<sup>21,22</sup> derivative spectrophotometry,<sup>23,24</sup> UPLC<sup>25</sup> and HPLC<sup>26,27</sup> methods. The quantification of REM from the biological samples was done using LCMS method.<sup>28</sup> REM along with metformin in formulations was estimated using spectrophotometry and HPLC methods.<sup>24</sup> Recently, Mandale *et al.*,<sup>29</sup> used RP-HPLC to determine VIL and REM from formulations simultaneously. However, the claimed approach has flaws, such as the lack of an internal standard and a considerable analysis time. As a result, the goal of this work was to establish a fast

and reliable analytical method for determining VIL and REM simultaneously utilizing linagliptin as an internal standard.

## MATERIALS AND METHODS

Vildagliptin (VIL), remogliflozin etabonate (REM), and linagliptin (LIN) were bought from Biochemix Limited as reference standards (Hyderabad, India). Sigma Aldrich provided analytical grade sodium dihydrogen phosphate and HPLC grade solvent acetonitrile (St. Louis, USA). The analytical grade ortho phosphoric acid used to modify the pH of the mobile phase was acquired from Scharlab (Sentmenat, Spain). Throughout the studies, Milli Q purified water (Milli pore, Burlington, MA, USA) was utilized. Before injecting into the HPLC instrument, the mobile phase was filtered with a nylon filter 0.45 m cellulose filter and the samples were filtered with a 0.22 m nylon syringe filter. Agilent HPLC apparatus (1200, Agilent Technologies, Waldbronn, Germany) with auto sampler (G1329A), degasser (G1322A), quaternary pump (G1311A), and diode array detector was used to separate analytes (G1315B). Chemstation software was used to monitor the chromatograms (Agilent Technologies, USA, Ver B 2.4.3). The analytes were separated using a Zorbax (100 mm x 4.6, 5 m, i.d) C<sub>18</sub> HPLC column. The pH of the mobile was measured using pH meter (Martini Instruments, Gallarate, Italy).

### Preparation of standard solutions

Required quantity of VIL was weighed and dissolved in water, while REM and LIN were dissolved in methanol to obtain 1000 µg/mL solutions. Solutions for calibration curve and validation were prepared by diluting the aforesaid solutions with mobile phase.

### Preparation of sample solution

Marketed formulation consisting of VIL (50 mg) and REM (100 mg) was obtained from the market. Twenty tablets were weighed and average weight was calculated. Tablets were powdered and a sufficient amount of powder, equivalent to 5 mg of VIL and 10 mg of REM, was weighed, diluted in 5 ml of methanol by sonication, and filtered into a 10 ml volumetric flask to prepare the sample solution for analysis. Fresh methanol was used to wash the residue, and methanol was added to adjust the final volume.

### Chromatographic conditions

The optimal liquid chromatographic conditions for the simultaneous determination of VIL and REM were

Zorbax C<sub>18</sub> HPLC column (100 mm x 4.6 μ, 5 μm, i.d) with acetonitrile and sodium dihydrogen phosphate (20 mM) in a ratio of 55 : 45 v/v at pH 5. On daily basis, mobile phase was prepared, filtered, and degassed using sonication. For the analysis, mobile phase was pumped at a flow rate of 1.2 mL/min and 20 μL of analyte solutions were injected. UV absorbance at 210 nm was used to monitor the chromatogram. All of the experiments were carried out at room temperature.

### Validation of HPLC method

The devised HPLC technique was validated using all three analytes in accordance with the ICH Q2 guidelines.<sup>30</sup> System suitability, linearity, sensitivity, precision, accuracy, stability, and robustness were all investigated as validation parameters.

### System suitability test

Standard solution containing 10 μg/mL, 20 μg/mL and 10 μg/mL of VIL, REM and IS respectively were evaluated under optimized HPLC conditions to assess system suitability. The analysis was carried out in five replicates, and the tailing factor, peak area, theoretical plate, resolution, and retention time were all calculated as system suitability characteristics.

### Linearity

The analytical method's linearity is defined as the direct proportionality of the analyte concentration in the sample to the result. VIL and REM standard solutions were made by transferring enough stock solution to achieve final concentrations of 10, 20, 30, 40, 50, and 60 g/mL of VIL and 10, 20, 40, 60, 80, and 100 g/mL of REM, respectively. The final concentration of 10 μg/mL of internal standard was maintained in all solutions. The peak area ratios of VIL to IS and REM to IS were computed from the corresponding chromatograms at various concentrations, and a linearity curve was plotted. Using Microsoft Excel, the slope, intercept, and regression coefficient were calculated.

### Sensitivity

The HPLC method's sensitivity was assessed by measuring the limit of detection (LOD) and limit of quantitation (LOQ). The LOD and LOQ were computed by multiplying the signal to noise ratio of the chromatogram by 3.3 and 10 times, respectively.

### Precision

The closeness of data acquired using successive sampling of the same solution under optimized experimental circumstances is demonstrated by the precision of

the analytical procedure. The method's precision was evaluated by injecting three distinct concentrations (low, medium, and high) in the linearity range to determine intraday and inter-day precision. The solutions were prepared with VIL concentrations of 10 μg/mL, 30 μg/mL and REM concentrations of 60 μg/mL and 10 μg/mL, 50 μg/mL and 100 μg/mL as well as a 10 μg/mL of internal standard in each solution. For intraday precision, solutions were injected on the same and for inter day same solutions were injected on three days in a row. The results were expressed as a percentage relative standard deviation after each solution was injected in triplicate.

### Accuracy

Accuracy of the analytical method reveals the correctness of the measurements. Standard addition method with percentage recovery was adopted for the assessment of accuracy. The accuracy study was done by adding three different concentrations (80%, 100% and 120%) of standard analytes at to the previously tested sample solution. The results were expressed as % recovery and percentage relative error for each solution, which were injected in triplicate.

### Stability Studies

Stock solution prepared was stored in the refrigerator and the working standard solutions were prepared on daily basis. Hence, the bench top and long storage stability of analytes were tested by evaluating the sample after 24 hr by three freeze-thaw cycles and after seven days of storage in the refrigerator (2-8°C). The findings of the analysis were compared between the fresh solutions and results after 24 and seventh day.

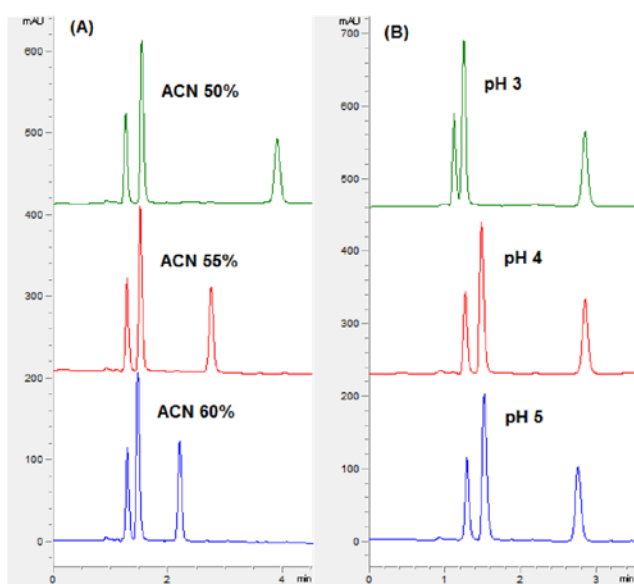
### Robustness

The reliability of an analysis with slight but deliberate changes in the experimental conditions was assessed by robustness study. Robustness study confirms the validity of analytical procedure even in slight changes in the experimental conditions. In HPLC, the parameters considered for the robustness includes, mobile phase composition ( $\pm 2$  mL acetonitrile) and pH ( $\pm 0.1$ ), detection wavelength ( $\pm 2$  nm), and flow rate ( $\pm 0.1$ ml). To investigate the influence of simultaneous changes in the experimental conditions, a central composite design, a sort of multivariate technique based on three level factor interaction studies, was used. Nineteen runs were completed in random sequence, including three center point experiments, as advised by the software (Design Expert Ver. 12). The percentage assay of analytes was

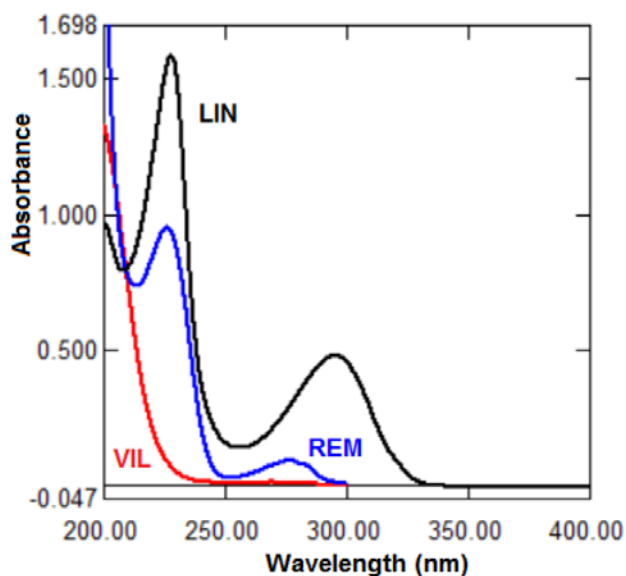
considered as the response to the experiments and Pareto chart was created using Design expert software.

### Optimization of HPLC method

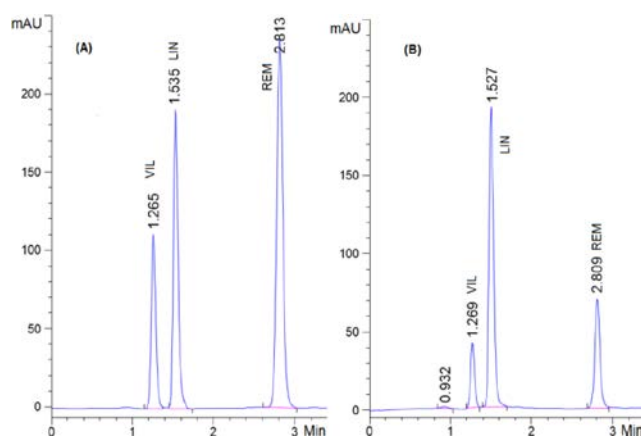
The separation of analytes was accomplished using commonly used  $C_{18}$  HPLC column (Zorbax 100mm X 4.6 mm, 5- $\mu$ m i.d). Various mobile phases such as acetonitrile, methanol, and phosphate buffer were used to optimized the HPLC process. Good separation was achieved with phosphate buffer and acetonitrile. Furthermore, the mobile phase's composition and pH were optimized by altering the amount of acetonitrile and the pH between 3 and 5. The retention period of analytes decreased as the amount of acetonitrile in the mobile phase increased. All three analytes eluted in 2.5 min with 60% acetonitrile, but VIL and LIN peaks overlapped at high concentrations. With 50% of acetonitrile analysis time was longer, REM was eluted after 3 min. With 55% of acetonitrile good base line separation was achieved between VIL and LIN, and REM was eluted within 3 min (Figure 2A), hence 55% acetonitrile was selected for further studies. The pH of the mobile phase was also optimized, with decrease in pH, resolution between VIL and LIN was decreased. At pH 5, however, all three analytes were separated with good resolution in less than 3 min (Figure 2B). The retention of REM was not impacted by the change in pH of the mobile phase. The concentration of sodium dihydrogen phosphate had no effect on the retention of all three analytes, however 20 mM phosphate buffer produced nice peak shapes. With a flow rate of 1.2 ml/min at ambient temperature,



**Figure 2: Chromatogram showing effect of percentage of acetonitrile (A) and pH of mobile phase (B).**



**Figure 3: UV absorption spectra of VIL, REM and LIN.**



**Figure 4: Typical chromatogram of standard solutions VIL, REM and IS (A) and formulation (B).**

the optimum mobile composition of 20 mM sodium dihydrogen phosphate at pH 5 and acetonitrile in the isocratic ratio of 45:55 v/v was determined to be the best suitable for getting well-defined peaks of all three analytes. A UV spectrum of three analytes was recorded to determine the detection wavelength, and the overlay spectra is shown in Figure 3. REM and LIN displayed maximum absorption at 225 and 228 nm, respectively, whereas VIL had no absorption at these wavelengths; nevertheless, all three analytes had high absorption at 210 nm. In order to have the best detector response for all three analytes, the ideal wavelength for detection and quantification was chosen as 210 nm. Figure 4A shows a typical example chromatogram of standard solutions of all three analytes.

**Table 1: System suitability and Validation parameter results of the proposed RP HPLC method for simultaneous determination of VIL and REM.**

Validation Parameters	VIL	LIN	REM
Retention time ± SD	1.265±0.02	1.527±0.05	2.813±0.04
Peak area ± SD	159.8±3.18	816.45±14.6	462.4±6.32
Resolution	--	2.01	5.62
Theoretical plate ± SD	2580.64 ±46.78	3093.38 ±68.98	7485.71 ±126.35
Tailing factor	1.04	0.986	1.08
Linearity range (µg/mL)	10-60	--	10-100
Slope	0.0183	--	0.0274
Intercept	0.0041	--	0.0102
Regression coefficient (r <sup>2</sup> )	0.9987	--	0.9995
LOD (µg/mL)	1.54	--	0.86
LOQ (µg/mL)	4.69	--	2.61
Accuracy (Mean %±%RE)	99.57±1.23	--	100.59±0.95
Precision			
Intra day	1.57	--	0.86
Inter day	0.93	--	1.42

### System suitability test results

The parameters of the system suitability test were determined by assessing both analytes in five replications, and the results were listed in Table 1. Standard deviation of retention time and peak area are well within the acceptable range. The theoretical plate for all three analytes was greater than 2000, and peak resolution was observed between the analyte peaks were greater than 2. The peak area and retention duration standard deviations were both within acceptable limits. For all three analytes, the tailing factors were about 1.

### Linearity

The linearity of the analytical method is defined as the direct proportionality of the analyte concentration in the sample to the response. Six standard solutions of VIL (10-60 µg/mL) and REM (10-100 µg/mL) along with 10 µg/mL of LIN as internal standard were analyzed in triplicate to create the calibration curve. The calibration curve was constructed by plotting the concentration against peak area ratio (analyte/IS). With a decent regression coefficient ( $R^2 \geq 0.998$ ), good linearity was obtained in the concentration ranges of 10 to 60 g/mL and 10-100 g/mL of VIL and REM, respectively (Figure 5).

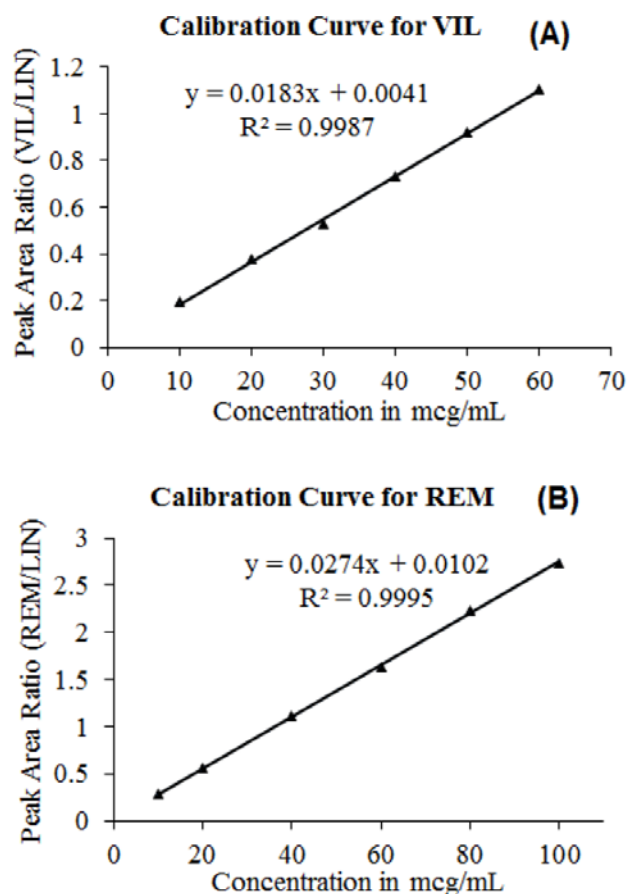


Figure 5: Calibration curves for VIL(A) and REM(B).

### Sensitivity

In Table 1, the LOD and LOQ values were recorded. The HPLC method's sensitivity was proven by the low LOD and LOQ concentrations.

### Precision

In terms of intraday and interday precision, the precision of the optimized HPLC method was determined. For VIL and REM, the % RSD computed from three measurements taken on the same day for intraday precision was 1.57 % and 0.86 %, respectively. The interday precision of the same solutions evaluated for three days revealed % RSD 0.93 % and 1.42 % for VIL and REM, respectively (Table 1), suggesting good precision of the HPLC method.

### Accuracy

The standard addition method was adopted for the evolution of accuracy of the HPLC method. The mean percentage recovery of three distinct analyte concentrations of ranged from 99.57 % for VIL and 100.59% for REM. The percent relative error for VIL and REM was found to be 1.23% and 0.95% respectively

Table 2: Assay and statistical calculation results.					
Formulation Concentration		HPLC method (Mean %± SD)		Ref method <sup>24,33</sup>	
REM	VIL	REM	VIL	REM <sup>a</sup>	VIL <sup>b</sup>
100 mg	50 mg	99.58 ± 0.58	99.33 ± 0.92	99.53 ± 1.08	100.08± 0.91
Student <i>t</i> -Test(2.306)		0.083	1.28		
F (6.388)		3.401	1.017		
Standard addition method					
Amount Added (µg/mL)		(% Recovery)			
40	20	98.85	99.28	101.26	100.39
50	25	101.46	98.37	99.81	99.08
60	30	100.87	100.67	99.44	101.75
Across Mean		100.39	99.44	100.17	100.40
%RSD		1.36	1.15	0.96	1.33

<sup>a</sup> HPLC method using Agilent XBD column (5 µm, 150 mm × 4.6 mm i.d.), and mobile phase acetonitrile-potassium dihydrogen phosphate (10 mM) 85:15 v/v, flow rate of 1 mL/min, at 210 nm<sup>33</sup>

<sup>b</sup> HPLC method using monolithic column (5 µm, 50 mm × 4.6 mm i.d.), and mobile phase acetonitrile and mixture of 25 mM sodium dodecyl sulfate and 10 mM potassium dihydrogen phosphate (pH 3.5) (4:58 v/v), 2ml/min, 230 nm<sup>24</sup>

<sup>c</sup> and <sup>d</sup> are critical values of *t* and *F*, respectively at *p*=0.05

(Table 1) which are well within the acceptable range (Percentage recovery 100±2% and %RE ≤ 2). The standard addition procedure was used to confirm the accuracy. (Table 2).

### Stability studies

Fresh solutions, 24 hr after preparing the working standard, and solutions stored in the refrigerator for seven days showed no change in the test results, demonstrating that the solutions are stable even after seven days.

### Robustness

To determine the influence of simultaneous changes in the experimental settings, a robustness study was conducted using a central composite design.<sup>31,32</sup> The Pareto charts (Figure 6) created with Design Expert software describe the significance of variable parameters. The parameters above the Bonferroni Limit are highly significant while the effects above the *t*-value are also significant and below the *t*-value are not significant. The combined effect of flowrate and wavelength had significant influence on the assay of VIL; however, the combined effect of acetonitrile percentage and wavelength had substantial effect on the REM assay, hence, need to be controlled carefully. However, individual parameter had no significant effect on the percentage assay of both the analytes, showing the robustness of the HPLC method.

### Application of proposed HPLC method for formulation

The optimized HPLC method was applied for the simultaneous determination of VIL and REM from the solid dosage form. The assay results are in agreement

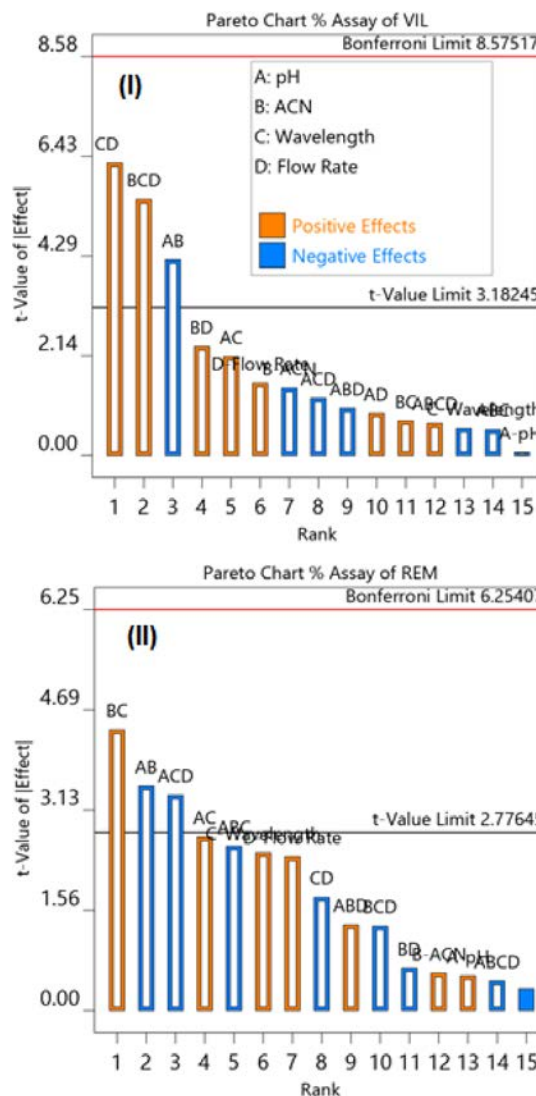


Figure 6: Pareto charts for VIL (I) and REM (II) showing robustness study results.

with the amount of analytes in the formulation. Further, tablet excipients did not show any peaks at the analytes peaks (Figure 4B) indicating absence of excipients interference. Furthermore, the percentage recovery determined by standard addition method confirmed the accuracy of the optimized HPLC method. In addition the assay results of the proposed HPLC method was compared with the reported methods.<sup>24,33</sup> The outcomes of the proposed and reported methods showed acceptable results in terms of accuracy and precision. The calculated students *t*-test and *F* test values for VIL and REM assay were less than the critical values.

## CONCLUSION

For the simultaneous quantification of VIL and REM from the solid dosage form, a rapid reverse phase HPLC method was developed. The proposed HPLC method was optimized in terms of mobile phase composition and pH for rapid and accurate separation of all three analytes with acceptable suitability parameters. The method was validated according to ICH Q2 guidelines, confirming the accuracy, precision and sensitivity. Furthermore, multivariate technique performed using three level factor interaction analysis supported the robustness of the HPLC method. The proposed approach was compared to the reported methods, and no significant differences in the assay findings were found. Because the proposed method is simple and quick, it may be used to conduct a quality control study on a VIL and REM-based formulation and accelerate release time of the final dosage form.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

**IS:** Internal Standard; **%RE:** Percentage Relative Error; **%RSD:** Percent Relative Standard Deviation; **ICH:** International Conference on Harmonisation; **DDP-4:** dipeptidyl peptidase-4; **SGLT-2:** Sodium glucose cotransporter-2; **HPLC:** High Performance Liquid

Chromatography; **UPLC:** Ultra Performance Liquid Chromatography; **LCMS:** Liquid Chromatography Mass spectrometry; **HPTLC:** High Performance Thin Layer Chromatography.

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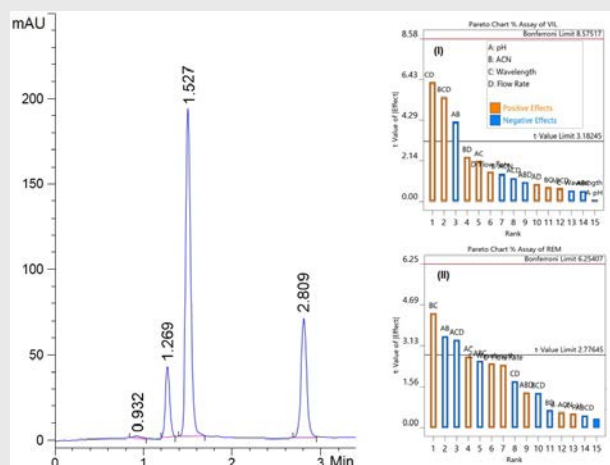
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## SUMMARY

Combination of vildagliptin and remogliflozin etabonate showed better glycemic control with added advantages hence, useful for the treatment of the type 2 diabetes. A simple, rapid, RPHPLC method was developed and validated for the concurrent quantitative analysis of both the analytes. Both methods showed linearity concentrations in the range of 10-60 µg/ml for VIL and 10-100 µg/ml for REM. The low LOD and LOQ found for REM and VIL by both methods indicated the good sensitivity of the methods. The good percentage recovery along with low percent relative error confirmed the accuracy of the methods. The percentage RSD for intra and inter-day precision was less than  $\pm 2$  %. Further, multivariate technique performed using three level factor interaction study confirmed the robustness of the HPLC method. The comparison of proposed method with the reported methods confirmed that no significant difference was observed in the assay results. As the proposed method is simple and rapid, can be used for the quality control study for formulation consisting of VIL and REM and accelerate release time of the final dosage form.



## PICTORIAL ABSTRACT



## About Authors

**Dr. Mahesh Attimarad** is graduated from Al-Ameen College of Pharmacy and completed his doctorate in Pharmaceutical and Analytical Chemistry from Rajiv Gandhi University of Health Sciences, Bangalore, India. He teaches Pharmaceutical Organic Chemistry, Pharmaceutical Analytical chemistry and Medicinal Chemistry courses at College of Clinical Pharmacy, KFU. He is actively involved in the research and received few research grants from KFU. His major research interests include Development of new analytical methods for drug molecules for estimation in pharmaceutical formulations and body fluids (blood and urine), Microwave assisted synthesis of organic compounds, and Design and synthesis of NSAIDs and Screening for anti-inflammatory and analgesic activities.

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