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RP-HPLC METHOD DEVELOPMENT AND VALIDATION OF REMOGLIFLOZIN ETABONATE, VILDAGLIPTIN AND METFORMIN HYDROCHLORIDE

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ABSTRACT
The present study aimed to advance a reliable and efficient RP-High-Performance Liquid Chromatography method for the simultaneous determination of Remogliflozin Etabonate, Vildagliptin, and Metformin Hydrochloride in both bulk drug samples and combined pharmaceutical formulations. Another objective was to indorse the developed analytical method for its applicability in routine quality control testing.
Methodology: Analysis by Chromatography was carried out using a Waters C-18 extended column (250 mm × 4.6 mm, 5 µm) as the stationary phase. The mobile phase consisted of Acetonitrile and Trifluoroacetic Acid buffer (pH 2.0) in the ratio of 65:35 (v/v). Prior to use, the mobile phase was degassed by sonication for 10 minutes and filtered through a 0.45 µm membrane filter. The separation was executed at a flow rate of 1.0 mL/min, while the temperature of the column was sustained at 25 ± 1°C. Detection of analytes was achieved using an Ultra Violet detector set at a wavelength of 215 nm.
Results: Under optimized chromatographic conditions, Metformin Hydrochloride, Vildagliptin, and Remogliflozin Etabonate were eluted at retention time of 2.05 min, 3.007 min, and 5.290 min, respectively, demonstrating effective separation of the three compounds.
Conclusion: The RP-HPLC method proposed was successfully validated using as per the ICH recommendations. Linearity, accuracy, precision, specificity and robustness of validation were within acceptable limits. The developed method was rapid, precise, and reproducible and was observed suitable for routine measureable assay of Remogliflozin Etabonate, Vildagliptin and Metformin Hydrochloride in pure and dosage form.

KEYWORDS: Remogliflozin, Vildagliptin, Metformin, Accuracy, Precision

1. INTRODUCTION

Hyperglycaemia is a metabolic illness that has various risk factors and that is characterized by a state of chronic hyperglycemia due to impairment in insulin sensitivity, insulin secretion or both. The worldwide epidemic of diabetes has made combination therapies, which correct various different underlying defects in glucose homeostasis, desirable. Remogliflozin Etabonate is an orally active SGLT2 inhibitor which decreases glucose reabsorption by the kidney to increase its excretion in the urine, thus reducing blood glucose[1]. Metformin hydrochloride, an initial drug in antidiabetic therapy, is based on a primary mechanism of action consist of a decrease in the production of glucose in the liver coupled with increase in peripheral utilization of glucose [2]. Vildagliptin is a DPP-4 inhibitor, which leads to an increase in endogenous concentrations of incretin hormones that increase glucose-dependent insulin secretion and decrease glucagon [3].

It can be concluded that the combination therapy of Remogliflozin, Metformin, and Vildagliptin provides a holistic treatment approach to attain better glycemic control in T2DM patients. There are needs to investigate an analytical procedure that will be validated for simultaneous determination of these three drugs with high accuracy and precision, because of the increase in the use of this combination in pharmaceutical formulations. This kind of method is especially significant in the pharmaceutical quality assurance, regular dosage form analysis and stability studies for product development and commercialization.

Fig 1: Metformin

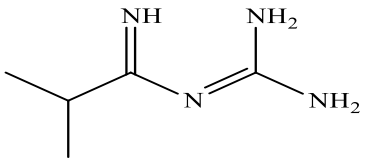


Fig 2: Vildagliptin

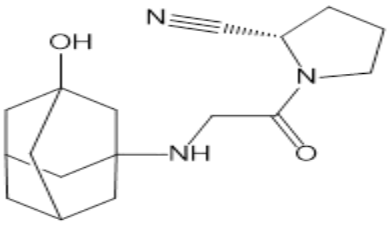
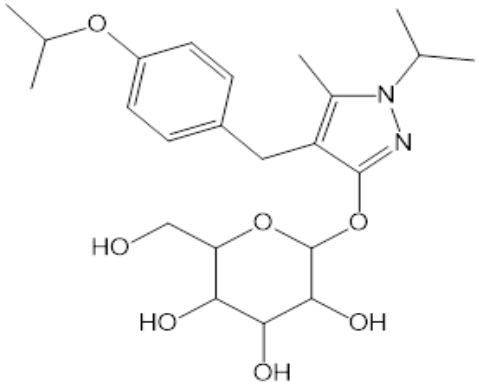


Fig 3: Remogliflozin



2. METHODOLOGY

2.1 Reagents and Chemicals

All analytical grade substances and chemicals were used in the study, the triflouroacetic acid and acetonitrile were obtained from, Merck.Ltd., Mumbai. Millipore purified using highly purified system Milli-Q purification system. The millipore water was used for preparation of mobile phase solutions. Very Glenmark Pharmaceuticals Ltd Remo MV 500 (Remogliflozin Etabonate 100mg, Vildagliptin 50mg, Metformin 500mg) was bought from local Pharmacy.

2.2 Instrumentation

The Agilent 1120 Compact LC HPLC system is equipped with an LC pump and a variable wavelength UV detector that is programmable. This system includes high pressure pump (HP) and gradient detector. The dual channel EZ Chrome Elite software was used for the acquisition, storage and evaluation of the chromatographic data. An extended Waters C18 column (250x5x4.6 mm) was chosen for the chromatographic separation.

2.3 Chromatographic requirements

The mobile phase system (acetonitrile: trifluoroacetic acid pH 2.0, 65:35 v/v) was determined as adequate composition for the successful chromatographic separation which was degassed and filtered under vacuum filtration through 0.45 µm membrane filter. Separation was done at room temperature with mobile phase flow rate of 1.0ml/min at constant flow and the effluents were monitored continuously at 215nm.

2.4 Preparation of Solutions

2.4.1 Trifluoracetic Acid Preparation

Trifluoroacetic acid (TFA) was diluted to give a 0.1% solution in HPLC grade water from the concentrated solution. After the pH of the solution that was obtained was carefully adjusted at 2, the solution was then filtered using 0.45-micron membrane filter and sonicated for 10 minutes.

2.4.2 Mobile Phase Preparation

The merged solvents (acetonitrile and trifluoroacetic acid) were then intensively degassed for 10 min by sonicating in an ultrasonic water bath and later filtered under vacuum with 0.45-micron membrane filter. Solvent used for dilution was the one which was eluted out from the column.

2.4.3 Sample Preparation

The Stock Solution of Metformin was prepared in 100 mg of Metformin in 100 ml of Acetonitrile to find the Conc. of 1000 µg/ml. Then, 1.0 ml was pipetted from this solution and further dissolved in the 10ml mobile phase to get 100 µg/ml take 0.5ml, 1ml,1.5ml, 2ml, 2.5ml,3 ml and make up to 10ml with mobile phase (Acetonitrile and trifluoracetic acid buffer in 65:35 ratio) in 10 ml volumetric flasks. These 6 concentrations are sonicated and the removal of the water bubbles is attempted.

The stock solution of Vildagliptin is made up by dissolving 100mg of the drug in 100ml of Acetonitrile to make 1000µg/ml solution. From stock solution of Vildagliptin take 1ml in 10 ml of Acetonitrile from this take 1ml, 2ml, 3 ml, 4ml, 5ml, 6 ml and make up to 10ml with mobile phase (Acetonitrile and trifluoracetic acid buffer in 65:35 ratio) in 10 ml volumetric flasks. These 6 concentrations are sonicated to ensure that all the water bubbles are removed.

The stock solution of Metformin HCl is prepared by taking 100mg in 100ml of Acetonitrile which gives concentration of 1000 µg/ml concentration. From stock solution of Metformin HCl take 0.5ml, 1ml,1.5ml, 2ml, 2.5ml,3 ml and make up to 10ml with mobile phase (Acetonitrile and trifluoracetic acid buffer in 65:35 ratio) in 100 ml volumetric flasks. The sonication is performed for all the 6 concentrations in order to eliminate water bubbles.

2.5 Analytical Method validation

2.5.1 Linearity

Linearity is a property which indicates that the measurements obtained are linearly related to the concentration of the substance measured (analyte) in the sample being analysed. Five different concentrations of response were used to prepare and analyze the calibration curve using the least squares approximation of the linear equation. The linearity was evaluated and the results were presented in tabular form in a systematic order within the concentration range of 50- 300 µg/ml for Metformin hydrochloride, 10-60 µg/ml for Vildagliptin, and 5-30 µg/ml for Remogliflozin which showed a proper and accurate calibration curve.

2.5.2 Accuracy

Intra/Inter-day variability was assessed for three consecutive days to determine accuracy. The percent relative standard deviation (RSDPH) of the peak area across the variation was calculated and tabulated. The formulation studied was Remo MV 500 in which Remogliflozin Ethalonate was present in the dose of 100 mg and the Vildagliptin, Metformin present in the dose of 50mg and 500mg respectively manufactured by Glenmark Pharmaceuticals Ltd.

2.5.3 Specificity

The specificity of analytical methodology was validated using the resolution of two different peaks and was supported by the retardation parameters, specified by retention time, resolution and tailing factor. **2.5.4 Robustness and Ruggedness** Evaluation of robustness was carried out for flow rate and for constituents of mobile phase. In addition, the robustness was studied by preparing drug solutions and injecting them under various circumstances. **2.5.5 LOD & LOQ** The procedures followed to estimate LOD and LOQ are different, based on the measured response and the slope, according to the regulation and result is tabulated (systematically presented).[4-10]

3. RESULTS AND DISCUSSION

3.1 System suitability

Attention has been given to the evaluation of the system suitability parameters and they have been found to be distinct, demonstrating that there is no cross over thus no interference.

Table 1: System Suitability results

Parameter	Method			Acceptance Criteria
	Metformin Hydrochloride	Vildagliptin	Remogliflozin	
Theoretical plates number per meter	3290	2931	3625	NLT 2000
Tailing Factor	1.201	1.428	1.088	NMT 2
Resolution	2.226		4.899	

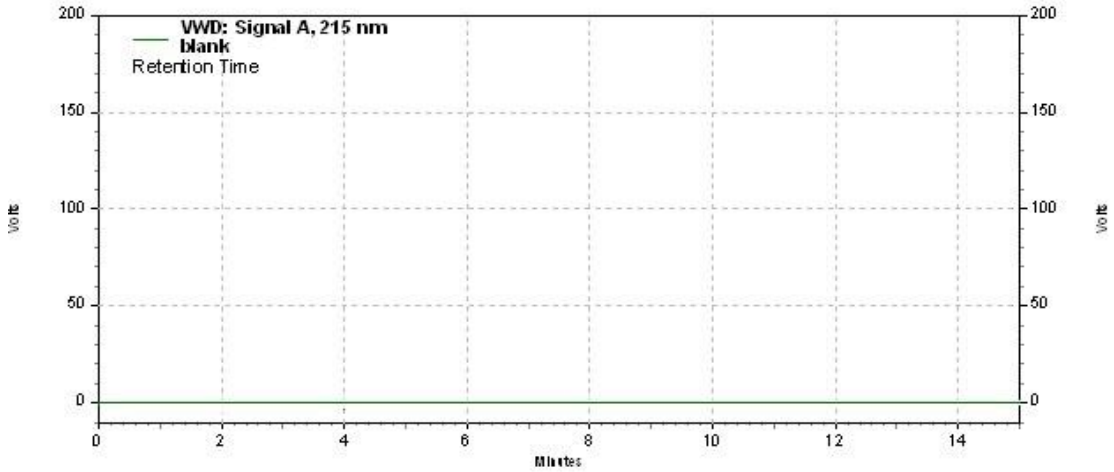


Fig 4: Chromatogram representing Blank run

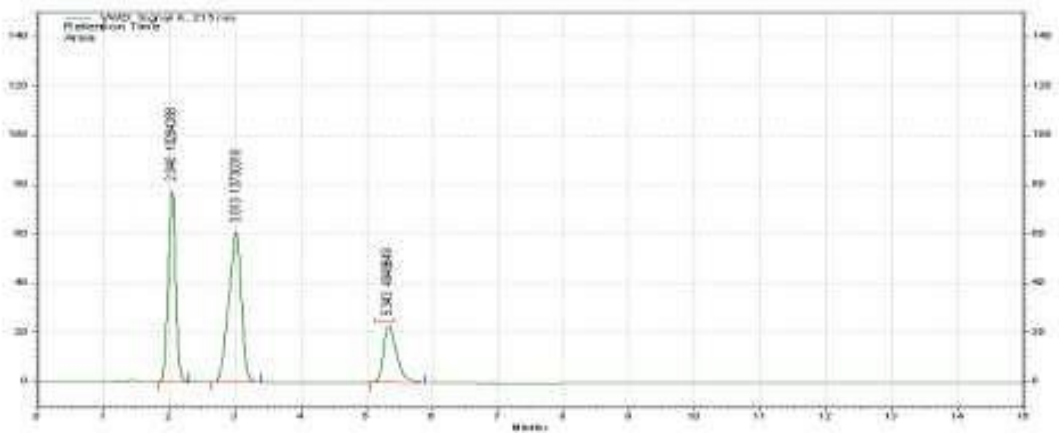


Fig 5: Chromatogram for optimised method

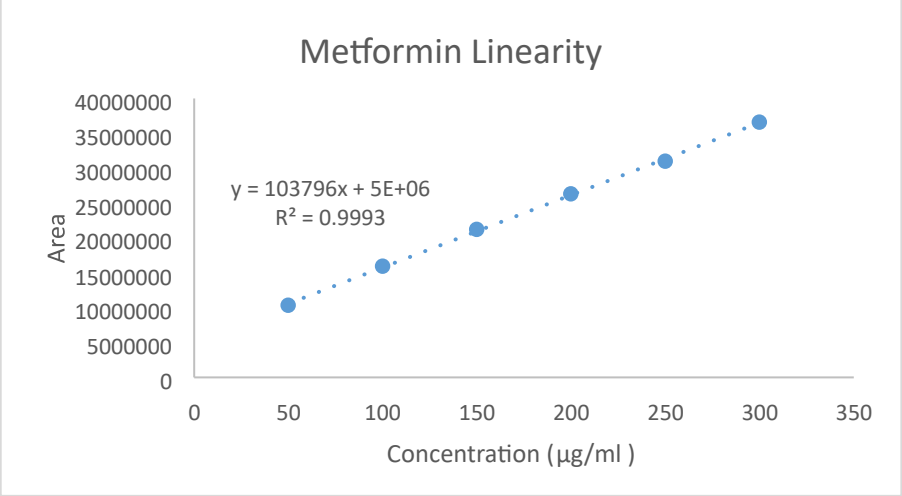
3.2 Linearity and Range

The linearity ranges were investigated for concentrations 50 – 300µg/ml Metformin hydrochloride, 10 – 60µg/ml Vildagliptin and 5 – 30 µg/ml Remogliflozin. A graph was used to show the linear relationship between the concentrations and Peak area for linear regression analysis. The regression coefficients R2 were found to be 0.9993 for Metformin, 0.9942 for Vildagliptin and 0.9965 for Remogliflozin which were found to be in good agreement in with the permissible limits proposed by the ICH.

Table 2: Linearity study results

S.No	Remogliflozin		Vildagliptin		Metformin	
	Conc(µg/ml)	Peak Area	Conc(µg/ml)	Peak Area	Conc(µg/ml)	Peak Area
1.	5	4949649	10	13730310	50	10313853
2.	10	9285665	20	20811206	100	15903626
3.	15	11334720	30	27754512	150	21226111
4.	20	15497390	40	33615969	200	26293364
5.	25	22329732	50	40092206	250	30976632
6.	30	31481928	60	49916901	300	36585163
R^2	0.9965		0.9942		0.9993	

Fig 6: Calibration Graphs of Metformin, Vildagliptin and Glimepiride



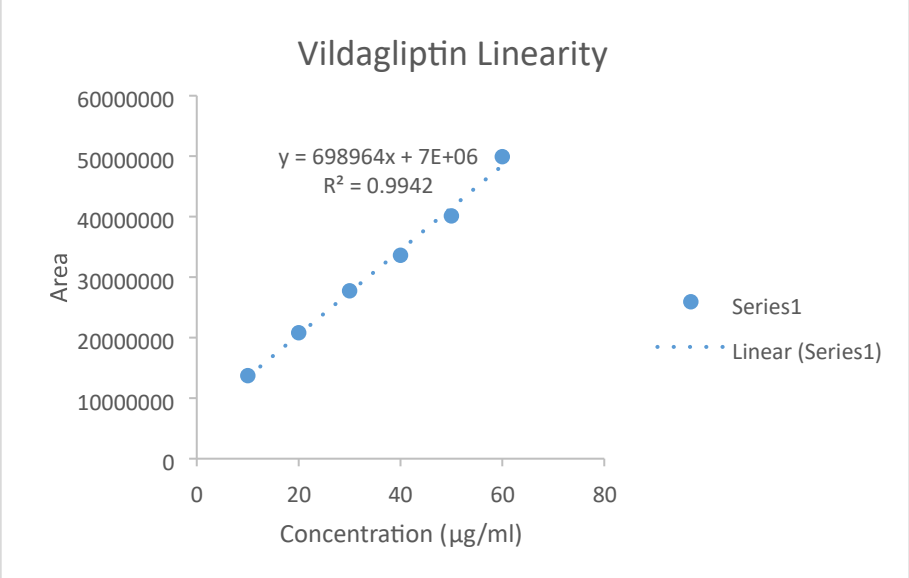
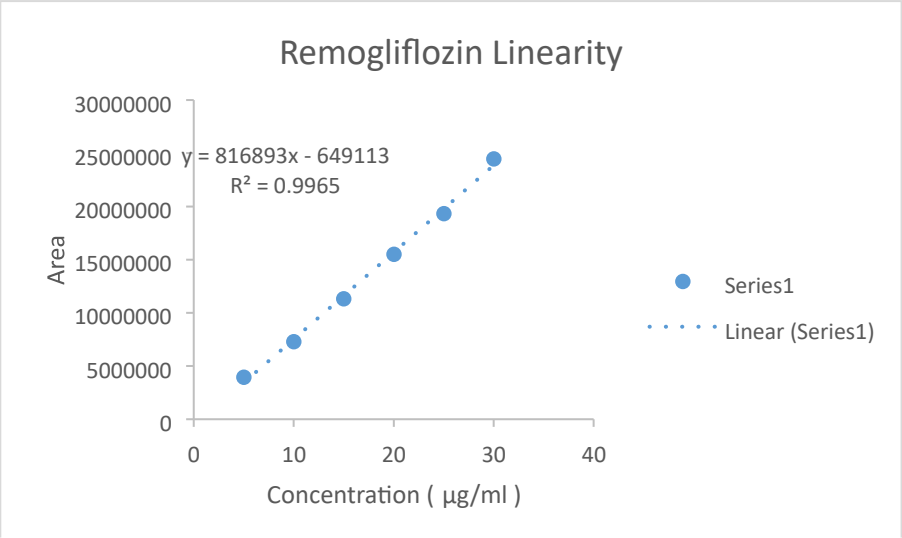


Fig 7: Graph for linearity studies



3.3 Precision

The precision of the method was evaluated by studying the intra-day and inter-day precision using a set of six replicates of pharmaceutical solutions with close monitoring of resultant responses.

Table 3: Intra Day Precision results

S. No	Remogliflozin Etabonate Area	Vildagliptin Area	Metformin HCl Area
1	15497490	33623451	26293214
2	15497354	33612349	26282354
3	15497690	33687230	26291394
4	15497890	33634583	26284563
5	15497790	33682104	38567834
6	15497990	33615639	38653403
Average	15495701	33642559	38665350
Standard Deviation	2338.727	33542.12	63848.06
% RSD	0.099	0.015	0.1

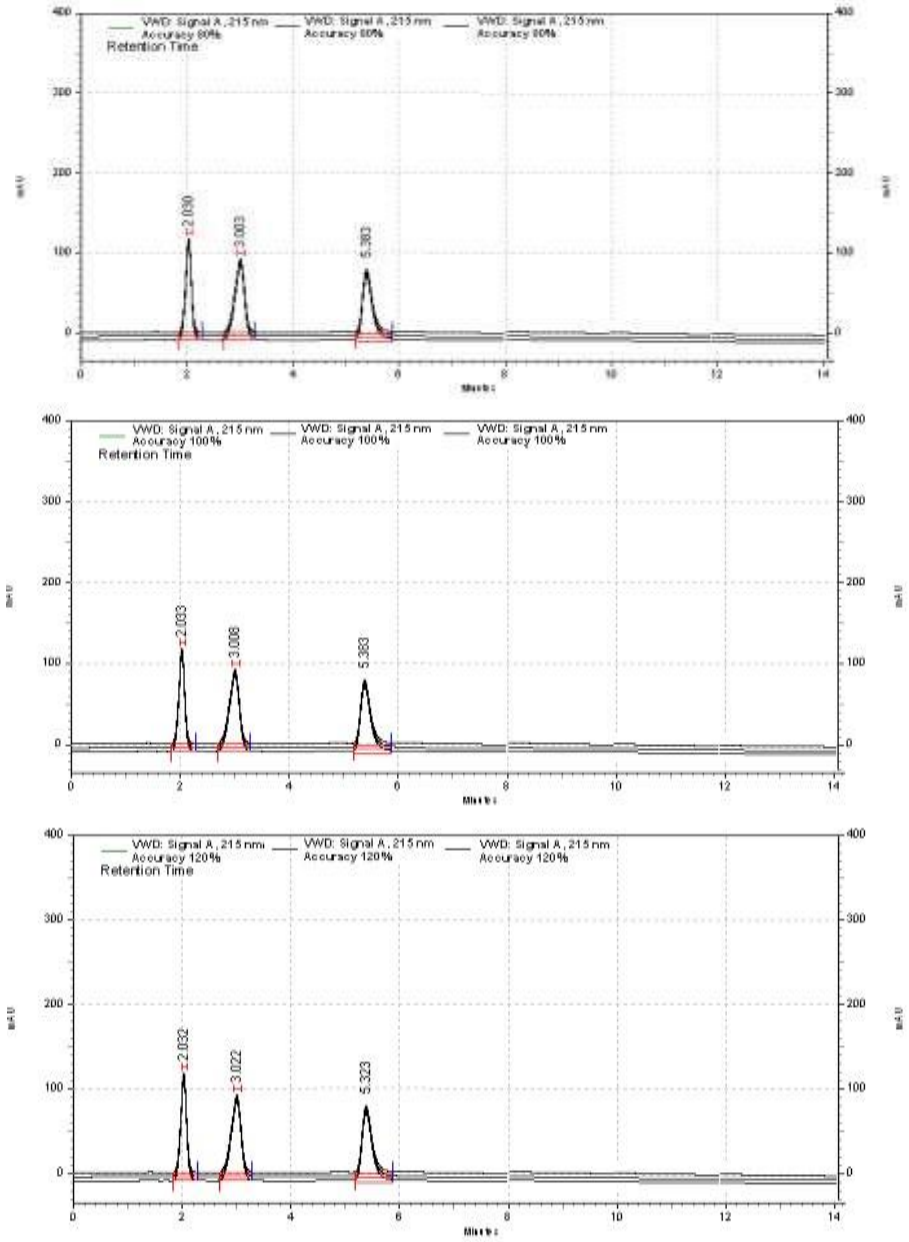
Table 4: Inter Day Precision results

S. No	Remogliflozin Etabonate Area	Vildagliptin Area	Metformin HCl Area
1	15497154	33616731	26291643
2	15497086	33665378	26296432
3	15497477	33667422	26292169
4	15497862	33681223	26299871
5	15497632	33687129	26299074
6	15497754	33687644	26294321
Average	15497494	33667588	26295585
Standard Deviation	317.6227	26688.4	3466.0191
RSD (%)	0.079	0.004	0.002

3.4 Accuracy

The accuracy of assay was determined by the standard addition method at different concentration percentage (80, 100 and 120%). Each preparation was carried out three times and analysed as per accepted procedures. The preparations were made in triplicate finally analysed according to the procedure. Recovery obtained for each spiked level (3, 10 and 30 ng/mL) for Metformin, Vildagliptin and Remogliflozin were 99.94-100.1, 99.9-100.01 and 99.97-100.63 % respectively which were indicating absence of any matrix interference.

Fig 7: Chromatograms for Accuracy



3.5 Strength

The imprecision associated with an analytical method is its intrinsic ability to be unaffected by small differences in chromatography. During the current investigation robustness was investigated by changing the flow rate of the mobile phase Flow Rate A by steps of ±0.1 ml/min and changing the Ph value of the mobile phase by steps of ±1 unit in the range of the optimized Ph value (Ph=7) without observing any significant deviations.

3.6 Limit of detection (LOD) and Limit of quantification (LOQ)

The LOD and LOQ were obtained according to the ICH guidelines and represented as a signal-to-noise ratio (S/N). S/N ratios of 3:1 and 10:1 were used to determine the LOD and LOQ, respectively. The LOD and LOQ of the analytical method were obtained to be 4.89 µg/ ml for Metformin, 2.905 µg/ ml for Vildagliptin and 1.411 µg/ ml for Remogliflozin, while 16.137 µg/ ml for Metformin, 9.586 µg/ ml for Vildagliptin and 4.653 µg/ ml for Remogliflozin as LOQ.

Table 5: LOD and LOQ results

Sample Name	LOD	LOQ
Metformin	4.896µg/ml	16.137µg/ml
Vildagliptin	2.905µg/ml	9.586µg/ml
Remogliflozin	1.411µg/ml	4.653µg/ml

4. CONCLUSION

This technique is unique in its excellent level of accuracy, precision, speed, sensitivity and selectivity. The separation of the pharmaceutical mixture is achieved using cost-effective solvents and the cleaning of apparatus is completed with the same type of solvents, making the method even more economically viable, for the quantification of the drug combinations

in both the purified form in bulk and in the tablet. The method developed was validated following the International Conference on Harmonization (ICH) recommendations. Any extra compound peaks were not observed that might interfere with the active ingredients and thus substantiating the purity of the compounds. Based on the above, it can now be concluded that the proposed method can be used for the routine quantification of the mentioned drugs in pharmaceutical dosage forms, bulk, drug monitoring and bioavailability studies.

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