Section A-Research Paper
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Studies on Octadysilane column and diol column to evaluate separation behaviour of some Antidiabetic drugs

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Abstract: Simultaneous and selective separation and retention of highly polar Metformin and its combination with other drugs such as moderately polar & hydrophobic drug molecules are difficult by using typical Reverse Phase –High Performance liquid chromatography. Metformin has a very short retention time when separated using RP-HPLC and it may co-elute with the plasma endogenous substances. Extremely polar antidiabetic drug like metformin (MET) along with some drugs often suffer from peak fronting/ tailing effects along with earlier elution with void volume in RP-HPLC. However, Normal phase chromatography is also not useful sometimes due to poor solubility of polar analytes in organic solvents. Therefore, alternative and complimentary technique to this RP-HPLC, is required for the separation of such extremely polar drug.Mixed mode chromatography can be better platform for separation of such types of drug molecules. The use of this newer technique of separations has been increased because of its advantages over traditional separation method for itsefficiency, resolving capacity, specificity, reproducibility and its robustness for lowerdrug concentration. The current work represents a comparative study on two developed and validated chromatographic methods for the simultaneous determination of the ternary mixture (MET, Remo and VIDA) in pure form and in combined tablet dosage. The first method is reversed-phase HPLC using Zodiac C18; column (5µ, 150 x 4.6 mm id)with a mobile phase consisting of 20M m Ammonium acetate buffer and methanol: Acetonitrile (10:90) V/V pH 3 at 230 nm. Chromatographic separation was achieved on an Acclaimed Mix Mode HILIC-1 column (150 mm × 4.6 mm, 5µm) applying an isocratic elution based on 20 mM ammonium acetate- acetonitrile (75:25, *v/v*) as a mobile phase.Both methods were fully validated following the ICH guidelines in terms of linearity, accuracy, precision, selectivity and robustness.until now no method has been reported for separation of Metformin, Ramogliflozin andVidagliptin.

Keywords: Analytical Method, Metformin, Ramogliflozin, Vidagliptin, Octadesylane Column, Diol Column, Antidiabetic Drugs, etc.

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Introduction: Several literatures have earlier reported the application of the most versatile C18 based chromatographic technique for the quantification of metformin (MET) from the pharmaceutical production. But in all tried C18 columns, metformin was too polar to be retained ,the polar metformin was not able to interact with lipophilic chains of the C18 stationary phase and hence it is difficult to retain metformin on conventional C18 columnsandmost negatively charged polar pharmaceutical amines do not retain in ion pairing mode[1,2]and, therefore, owing to their hydrophilic characteristics, it lowers their binding capacities to the ODS. Secondly, eventhough to encourage the drug–ODS interaction, the "ion suppression effects" with additional basic buffers further exhibit the peak tailing

or fronting effects[3] and, therefore, it disrupts the elution order and selectivity and hence for making effective separation some other analytical techniques were recommended and demonstrated which predominantly included the hydrophilic interaction liquid chromatography. HILIC, although not as well known or commonly used, has advantages over RP-HPLC in that both acidic and basic polar molecules can be retained[4,5]. Strong retention of polar molecules is possible and adjustments to mobile phase composition can greatly influence the desorption of analyte also it offers an interesting yet viable alternative to more widely used chromatographic techniques, such as normal phase chromatography, for the retention of polar molecules[1,2,3].

The Acclaim Mixed-Mode HILIC-1 column is a silica-based packing material that incorporates both Reversed-Phase (RP) and Hydrophilic Interaction Liquid Chromatography (HILIC) properties. Unlike either traditional RP or HILIC stationary phases, the packing features an alkyl long chain with a hydrophilic polar terminus, and demonstrates great potentials for separating a wide range of both highly polar and non-polar molecules, in either RP mode or HILIC mode.

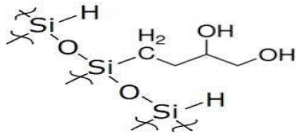

Fig.1: Diol Stationary Phase

Experimental

Instrumentation: The high-performance liquid chromatography (HPLC) of Shimadzu SCL-10A_{VP} inbuilt with binary pump (LC-10AT_{VP}), UV detector (SPD-10A_{VP}), Rheodyne 20µl loop capacity manual injector (P/N 77251) was used throughout the analysis. The LC-Solution software was used to interpret the HPLC reports. Zodiac C18 (150 x 4.6 mm. 3.5µ) and Acclaimed Mix-Mode HILIC-1 (5µm; 150 x 4.6 mm ID.) column was purchased from Ultrachrom Innovative Pvt. Ltd was used throughout the analysis. Digital weighing balance (ME-204) purchased from Mettler-Toledo (USA), ultra-sonicator Labman® purchased from UltraChrom Ltd, India. Digital pH meter from Mettler-Toledo was purchased from (Mumbai-India). 50 µ micro-syringe was purchased from Hamilton USA. 0.20µ and 0.45µ nylon membrane filters were purchased from Phenomenex® Mumbai, India.

Reagents and Reference Samples: The reference standards; Metformin, Remogliflozin and Vildagliptin were obtained as a gift sample from Yarrow Pharma Ltd. Ammonium acetate was purchased from Merck Ltd. (Mumbai-India) HPLC grade acetonitrile and deionised water were purchased from Merck (Mumbai, India). 0.20µ and 0.45µ nylon membrane filters were used and purchased from UltraChrom Innovative Pvt. Ltd. (India). All other chemicals and reagents were used of HPLC grade.

Standard Stock Solutions: Standard stock solutions of 1 mg/mL of standards, Metformin, Remogliflozin and Vildagliptin were prepared separately by dissolving 10 mg of the drug in 10 ml of acetonitrile-methanolwater (3:4:3 v/v) in a 20 mL volumetric flask. Furthermore, freshly prepared standards were mixed to get the concentration 100 ppm each for performing validation studies like repeatability, precision and robustness studies. Standard stock solution was then ultrasonicated for 10-20 minutes and filtered through 0.20µ nylon filters prior to the HPLC analysis.

Chromatographic Conditions: Chromatographic separation was achieved on a Acclaimed Mix Mode HILIC-1 column (150 mm × 4.6 mm, 5µm) applying an isocratic elution based on 20 mM ammonium acetate- acetonitrile (75:25, *v/v*) as a mobile phase. The ultraviolet detector was operated at 230 and 254 nm. The buffer solution was filtered through 0.2 µm nylon membrane filter and degassed for 10-20 min in an ultrasonic bath prior to its use. The mobile phase was pumped through the column at a flow rate of 1 mL min-1. The column temperature was adjusted to 28 C and the injection volume was 20 µL.

Sample Preparation for Drug Accuracy Studies: Exactly 5 tablets of valdiff-M consisting 500 mg of metformin and 50 mg of vildagliptin were weighed separately and the average weight wasdetermined. They were mixed and crushed to fine powder into the mortar and pestle. An accurately weighed amount of the finely powdered equivalent to 10 mg was dissolved in 10 ml of acetonitrile-methanol-water (3:3:4). It was then ultrasonicated and filtered through 0.45µ nylon filter. Furthermore, serial dilutions were made to get the final concentration 10 ppm of vildagliptin and equivalent to 100 ppm of metformin. The solution was then sonicated and analysed as mentioned in the experimental section

Sample Preparation for Linearity/Calibration studies: 1000 ppm (1000 µg/ml) of standard stock solution of remogliflozin, metformin and vildagliptin was made separately; then all three drugs' solutions were mixed in order to get 100 ppm of each in a homologous mixture. Subsequently, serial dilutions of five different concentrations such as 65 ppm, 31.50 ppm, 12.25 ppm, 6.25 ppm and 3.12 ppm were made, ultrasonicated and then analysed using the experimental section 5.4. Furthermore, the calibration curve (linearity graph) was plotted by calculating the peak area against known concentration to determine LOD, LOQ and regression coefficient (R^2) value.

Precision Studies of the Proposed Method: The homologous mixture of remogliflozin, metformin and vildagliptin of similar concentration 100 ppm each were analysed thrice within the same day (intraday precision) as well as, three successive days (intermediate precision) using the chromatographic condition mentioned in section 5.4 and then average, mean standard deviation and relative standard deviation (RSD) in percentage was calculate.

Robustness for the Chromatographic Method: The flow rate of the mobile phase was changed by 1.00 ±1 decimal from 1 mL/min to 1.1 mL/min and to 0.9 mL/min to evaluate the effect of the flow rate; similarly, the variation of organic modifier used as acetonitrile was changed by ±2% from 75% to 77% and 73% to monitor the peak area and retention time. Finally, the effect of wavelength was monitored by making deliberate variation from 230 to 232 and 228nm and the differences in various system suitability parameters including retention time, peak tailing, capacity factor, resolution and theoretical plates were tested and evaluated. Robustness study was performed as per the procedure mentioned under the experimental section 5.4.

Method validation procedures

PrecisionStudies: Precision results were expressed in relative standard deviation (RSD). In repeatability, standard stock solution of REMO,MET, VIDA of similar concentration (100 ppm) was injected six times a day and their resultant peak areas and RSD were determined. Similarly, in intraday and intermediate precision (three different days) the triplicate of standard stock solution containing 100 PPM of REM,VIDA & MET were injected thrice and their respective RSD were calculated.

Linearity and Range: Linearity was determined by using the calibration curve for the concentration between range of 1.25,2.5,5,10,20 µg/ml for Remo, 0.37, 0.75,1.25,2.5,5 µg/ml for VIDA & 6.25,12.5,25,50, 100 µg/ml for MET respectively . Linearity of peak area against the concentration was calculated to get regression values and correlation coefficient (r2).

Limit of detection (LOD) and limit of quantification (LOQ): Limit of quantification LOD and limit of quantification (LOQ) were determined by injecting the homologous mixture of REMO and MET,VIDA Furthermore, the LOD and LOQ were calculated using the following formula:

LOD = 3.3 × (Std. Deviation of intercept/slope)

LOQ = 10 × (Std. Deviation of intercept/slope)

Robustness : The robustness studies involved the small variations in selected separation parameters such as changes in temperature (±2°C), flow rate (±0.2 ml/minutes), and wavelength (±2 nm) were tested and evaluated. the flow rate of the mobile phase was changed from 1.1 $mL.min^{-1}$ to 1.3 mL.min-

[1] and 0.9 mL.min; the results derived were evaluated for any changes in capacity factor (k'), resolution (R), theoretical plates (N), and tailing factor.

Accuracy : The accuracy was determined by mixing the fixed concentration of standards, REMO (2.5 µg/ml) a,VIDA& MET (250 µG/ ml) with varying concentrations of REM-500 tablets as 2 µg, 2.5 µg, and 3 µg to make the 80%, 100%, and 120%, respectively. The analysis was performed in a triplicate with data calculated to determine the percentage (%) drug recovery, mean ± SD, and percentage (%) RSD.

Results and discussion

Several attempts were made on simultaneous analysis of metformin with either Vildagliptin or Remogliflozin but very few work were found on simultaneous analysis of all three drugs combination. Importantly, all separation previously performed by using reverse phase technique, consisting C_{18} column with different dimensions and specifications. Nonetheless, as realised in all attempted previous articles highly polar Metformin elute with t_0 in C_{18}. In addition, while considering simultaneous quantification together with ionic polar Vildagliptin and neutral polar Remogliflozin need moderately higher composition of organic modifiers such as methanol or acetonitrile. But, increasing initial volume of organic modifier increases the chances of early elution of Metformin. Another major limitation is Vildagliptin has low UV absorbance sensitivity (≈210 nm) whereas Metformin and Remogliflozin have around 210 nm, hence in simultaneous analysis considering isobastic point for their quantification is tentatively difficult. Moreover, considering the lowest wavelength for simultaneous quantification is not conducive for selected ammonium acetate since it exhibits unstable erratic baseline. Moreover, to improve the retention of Metformin while considering simultaneous analysis with selected drugs, the run time will be elongated in ODS phase.

Therefore, alternative to reverse phase chromatography, HILIC mode technique has been considered for simultaneous estimation of selected drugs. The details of all results were explained in further work.

Prior to the HPLC analysis, the UV analysis of all selected drugs was performed by selecting the wavelength from 350 – 200 nm. In results, both Remogliflozin and Metformin shows significant maximum absorbance at 210 nm wavelength. In contrast, the Vildagliptin has very low UV sensitivity which has optimum UV absorbance limit is 203-205 nm. However, the aqueous mobile phase with ammonium acetate has the UV absorbance strength more than 210 nm. Beside these, the real isobestic point for all drugs were lying between something around 250-260 nm wavelength. Therefore, two wavelengths; 230 and 254 were selected by applying PDA detector for HILIC chromatography (Figure 2).

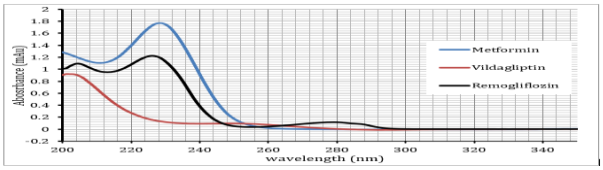

Fig: 2: Overlay UV spectra of Metformin, Vildagliptin and Remogliflozin

Method Development

Simultaneous Estimation by Using Octadesylane(C18) Column: When Simultaneous estimation of all 3 drugs(MET,VIDA & REM) wasCarried out by using C18 column at composition Solvent A; 20 Mm ammonium acetate B; MeOH-ACN (10:90 v/v) at wavelength 230 nm all three analytes were overlapped and Eluted with void volume(Fig. 2)(Table. 1) and at 205 nm,Metformin eluted at t_0 whereas Remogliflozin and Vildagliptin were eluted quite late (Fig. 3) (Table2).

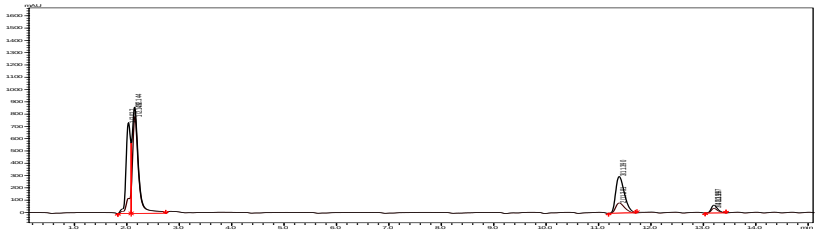

Fig. 3: Report of REM, VIDA and MET on C_{18} column at 205 nm gradient elution

Table 1: Trial for simultaneous analysis of MET, REM and VIDA

Peak#	Ret. Time	Area	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	2.027	4570203	8.6415	787.116	--	0	--	0
2	2.144	7084523	13.3957	932.32	0.411	0.058	--	0
3	11.39	3387695	6.4056	21518.21	31.264	4.619	1.444	80.035
4	13.197	534546	1.0107	52444.08	6.681	5.51	1.337	1.193

Simultaneous Estimation by Using Diol Column: When analytes are separated by using Diol columnall three selected analytes were well separated and follows all ICH guidelines.

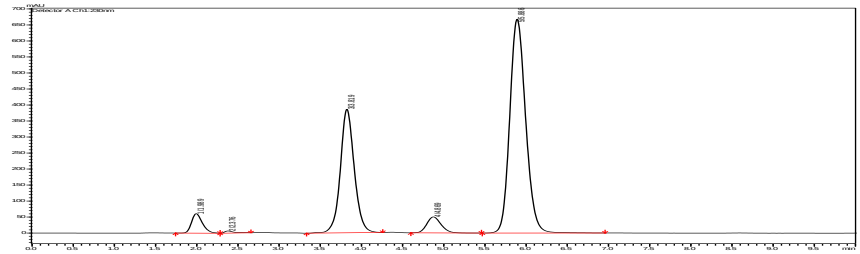

Fig. 4: Developed method for estimation of Remogliflozin, Vildagliptin and MetforminAcclaimed Mix Mode HILIC-1 column(Diol)

Table 2: Method for estimation of Remogliflozin, Vildagliptin and Metformin

Peak#	Ret. Time	Area	Height	Area %	T.Plate#	Resolution	k'	Tailing F.
1	1.989	560871	60781	3.8116	1012.448	--	0	1.332
2	2.376	51178	6771	0.3478	2410.588	1.742	0.194	2.043
Remogliflozin	3.819	4537971	385702	30.839	2452.875	5.749	0.92	1.085
Vildagliptin	4.869	600834	49864	4.0831	3601.997	3.318	1.448	1.188
Metformin	5.886	8964174	667980	60.9185	4381.955	2.991	1.959	1.176

System Suitability Tests for Remogliflozin, Vildagliptin and Metformin: The proposed simultaneous estimation of all three selected drugs; Remogliflozin, Vildagliptin and Metformin were tested to determine the basic separation factors of system suitability studies, including— theoretical plate (N), capacity/retention factor (k'), resolution (R), separation factor (α), tailing factor (*T*) and relative standard deviation (RSD). As resulted, after 6 successive injections of all selected drugs of similar concentration, all separation parameters were in accordance with ICH guidelines (7. 3).

Table3: System suitability studies of REM, VIDA and MET

System suitability parameters	REM	VIDA	MET
Theoretical plates (N)	2452	3601	4381
Capacity Factor (K')	0.92	1.44	1.95
Resolution (R)	---	3.31	2.99
Asymmetry/Tailing factor (T)	1.08	1.18	1.17
Retention time (t_R)	3.81 min.	4.86 min.	5.88 min.
Wavelength of Detection (nm)	210 nm	210 nm	210 nm
Repeatability (% RSD)	0.33	1.30	0.41
Intra-Day Precision (% RSD)	0.29 - 0.84	0.62 - 1.95	0.43 - 0.79
Inter-Day Precision (% RSD)	0.82 - 1.10	0.30 - 1.26	0.43 - 1.47
Linearity range	3.9 – 62.5 µg.ml^{-1}	3.9 – 62.5 µg.ml^{-1}	3.9 – 62.5 µg.ml^{-1}
Regression equation	Y= 43936x + 26959	Y= 5833.2x + 5055.1	Y= 88345x + 47830
SE of intercept (S_e)	4789.20779	3113.822334	8895.354744
SD of intercept (S_a)	11731.11536	7627.275868	21789.0802
Correlation Coefficient (R^2)	1	0.999	1
LOQ^a (µg. mL^{-1})	2.67 µg/ml	5.34 µg/ml	1.01 µg/ml
LOD^a (µg.mL^{-1})	0.80 µg/ml	1.60 µg/ml	0.30 µg/ml

Method validation studies: The proposed RP-HPLC method for the simultaneous quantification of Remogliflozin, Vildagliptin and Metformin was validated as per the ICH guidelines. The validation parameters likerepeatability, precision (interday/intermediate), robustness/ruggedness, linearity/calibration, force degradation and accuracy studies were tested and evaluated and found they are in accordance with the ICH guidelines.

Repeatability studies: Implementing the procedure under chromatographic condition of experimental section (5.3), the homologous mixture of 100 ppm of each selected analytes was injected six times with similar procedure within a same day. The % RSD was calculated and found it is less than 2% for Remogliflozin (0.33%), Vildagliptin (1.30%) and Metformin (0.41%).

Table 4: Repeatability data of REM, VIDA and MET

S. No.	Remogliflozin at 210 nm	Vildagliptin at 210 nm	Metformin at 210 nm
	Peak Area; Conc. 20 ppm	Peak Area; Conc. 5 ppm	Peak Area; Conc. 100 ppm
1	4237971	560834	8464174
2	4230084	580207	8475041
3	4213869	561320	8401334
4	4200808	564947	8421736
5	4234048	564161	8407204
6	4221243	570623	8476044
Mean	**4223003**	**567015.3333**	**8440922.167**
STD. DEV.	**13966.5256**	**7349.915011**	**34671.40728**
RSD (%)	**0.33**	**1.30**	**0.41**

Precision studies for REM, VIDA,and MET: The precision of HPLC method represents its closeness to the agreement among the series of repetitive results, derived after multiple sampling of the same homogenous mixture of selected drugs under the given conditions. For intermediate variability for precision studies, this method is significantly precise over the testing range of Remogliflozin, Vildagliptin and Metformin. Moreover, the peak area of all studied samples was also correlated with selected concentration since as observed their percentage relative standard deviation (RSD) was less than 2%. Thus, it reflects, the proposed method has acceptable precision with minimum variations and can be applicable for routine analysis.

Intraday and interday (intermediate) precision: Implementing the chromatographic procedure mentioned under experimental section (5.3), the homologous mixture of REM, VIDA and MET of three replicates of similar concentrations; were tested within a same day. The percentage RSDs for all three drugs was calculated and they were found less than 2%. The results were shown in Table 5to 7.

Table 5:Intraday precision data of REM

Drug Name: Remogliflozin				
S. No.	**Concentration (ppm)**	**Area**	**Mean ± SD**	**%RSD**
1	20 PPM	4237971	12288.45934	0.29
	20 PPM	4230084		
	20 PPM	4213869		
2	20 PPM	4200808	16765.31563	0.40
	20 PPM	4234048		
	20 PPM	4221243		
3	20 PPM	4490298	37533.234	0.84
	20 PPM	4451020		
	20 PPM	4526059		
Range of %RSD				**0.29 - 0.84**

Table 6: Intraday Precision data of VIDA

Drug Name: Vildagliptin				
S. No.	**Concentration (ppm)**	**Area**	**Mean ± SD**	**%RSD**
1	5 PPM	560834	11047.38351	1.95
	5 PPM	580207		
	5 PPM	561320		
2	5 PPM	564947	3525.909244	0.62
	5 PPM	564161		
	5 PPM	570623		

3	5 PPM	599450	7626.440257	1.26
	5 PPM	603526		
	5 PPM	614217		
Mean %RSD				**0.62 - 1.95**

Table 7: Intraday precision data of MET

Drug Name: Metformin				
S. No.	**Concentration (ppm)**	**Area**	**Mean ± SD**	**%RSD**
1	100 PPM	8464174	39790.44931	0.47
	100 PPM	8475041		
	100 PPM	8401334		
2	100 PPM	8421736	36284.71553	0.43
	100 PPM	8407204		
	100 PPM	8476044		
3	100 PPM	8918273	70393.48489	0.79
	100 PPM	8928364		
	100 PPM	9044930		
Mean %RSD				**0.43 - 0.79**

Interday (intermediate) Precision studies of REM, VIDA and MET: Implementing the chromatographic procedure mentioned under experimental section (5.3), the homologous mixture of REM, VIDA and MET of three replicates of similar concentrations (100 ppm) were tested and evaluated for three successive days (interday/intermediate precision). Furthermore, the percent RSD was calculated and found it is less than 2%; for all selected analytes in simultaneous HPLC-UV analysis (Table 8-10).

Table 8: Interday (intermediate) Precision data of remogliflozin

Drug Name: Remogliflozin				
S. No.	**Concentration (ppm)**	**Area**	**Mean ± SD**	**%RSD**
DAY 1	20 PPM	4490298	37533.234	0.84
	20 PPM	4451020		
	20 PPM	4526059		
DAY 2	20 PPM	4585701	37429.70483	0.82
	20 PPM	4586439		

	20 PPM	4521243		
DAY 3	20 PPM	4544530	49524.65625	1.10
	20 PPM	4451020		
	20 PPM	4526059		
	Range of % RSD			**0.82 - 1.10**

Table 9: Interday (intermediate) Precision data of Vildagliptin

Drug Name: Vildagliptin				
S. No.	**Concentration (ppm)**	**Area**	**Mean ± SD**	**%RSD**
DAY 1	5 PPM	599450	7626.440257	1.26
	5 PPM	603526		
	5 PPM	614217		
DAY 2	5 PPM	621967	1881.010455	0.30
	5 PPM	624338		
	5 PPM	620623		
DAY 3	5 PPM	617772	7414.895167	1.21
	5 PPM	603526		
	5 PPM	614217		
	Range of % RSD			**0.30 - 1.26**

Table 10: Interday (intermediate) Precision data of metformin

Drug Name: Metformin				
S. No.	**Concentration (ppm)**	**Area**	**Mean ± SD**	**%RSD**
DAY 1	100 PPM	8918273	70393.48489	0.79
	100 PPM	8928364		
	100 PPM	9044930		
DAY 2	100 PPM	8421736	36284.71553	0.43
	100 PPM	8407204		
	100 PPM	8476044		
DAY 3	100 PPM	9194615	133468.3649	1.47
	100 PPM	8928364		
	100 PPM	9044930		
	Range of % RSD			**0.43 - 1.47**

Linearity (Calibration) studies of REM, VIDA and MET: The linearity/calibration studies of HPLC-DAD method represent its ability to explicit the results that should proportional to the known concentration of studied analytes within the selected range of 62.5, 31.25, 15.75, 7.35 and 3.25 µg/ml against the peak area (mAu). Therefore, over the known concentrations of REM, VIDA and MET their corresponding area were found highly proportional since as noted their regression coefficients (R^2) were exactly 1 for Remogliflozin and Metformin and 0.999 for Vildagliptin (Figure 5-11 and Table 11- 14).

Furthermore, the limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the response and the slope of the regression equation; shown in Table 15-17.As observed, the LOD 0.80, 1.60 and 0.30 µg/ml and LOQ were 2.17, 5.34 and 1.01 for REM, VIL and MET respectively. These results signify that the selected wavelength 210 nm is more sensitive for Remogliflozin and Metformin and apparently less sensitive for Vildagliptin. However, this lower UV sensitivity was also observed for some other gliptin class of drugs including Evogliptin, Sitagliptin and Saxagliptin. Thus, the proposed method can be used for the routine HPLC analysis of either individual or simultaneous analysis of selected drugs from pharmaceutical drugs or biological fluids.

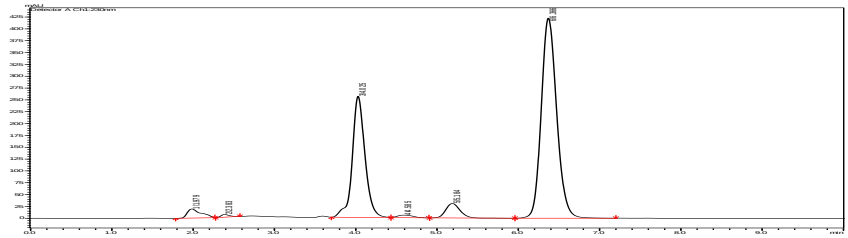

Fig. 5: Calibration studies of 62.5 ppm of REM, VIDA, and MET

Table 11: Calibration studies of 62.5 ppm

Peak#	Ret. Time	Area	Height	Area%	T. Plate#	Resolution	k'	Tailing F.
1	1.979	227904	19181	2.5133	801.551	--	0	1.697
2	2.383	41717	5664	0.4601	2234.824	1.68	0.204	1.404
3	4.025	2776398	255483	30.6182	3305.336	6.817	1.034	0.998
4	4.585	71185	5406	0.785	2810.686	1.789	1.317	--
5	5.184	370734	30623	4.0885	4012.818	1.78	1.62	1.199
6	6.366	5579866	422597	61.5349	5289.946	3.49	2.217	1.179

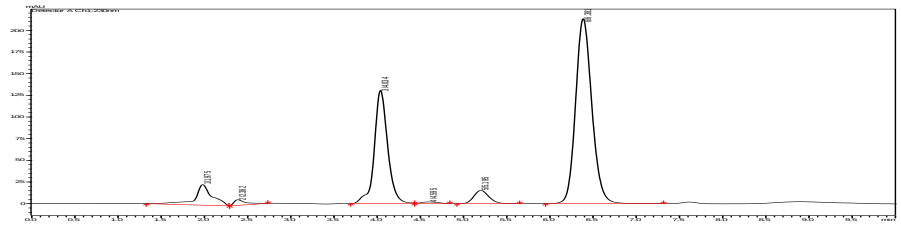

Fig. 6: Calibration studies of 31.25 ppm of REM, VIDA, and MET

Table 12: Calibration studies of 31.25 ppm

Peak#	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.
1	1.975	329070	23679	6.8435	929.236	--	0	0.824
2	2.382	63231	6496	1.315	1757.414	1.67	0.206	2.477

3	4.034	1403351	130428	29.1849	3435.71	6.577	1.042	0.989
4	4.595	24864	1824	0.5171	2588.834	1.76	1.326	--
5	5.195	183959	15250	3.8257	4107.667	1.753	1.63	1.19
6	6.381	2804013	213443	58.3138	5397.234	3.53	2.23	1.168

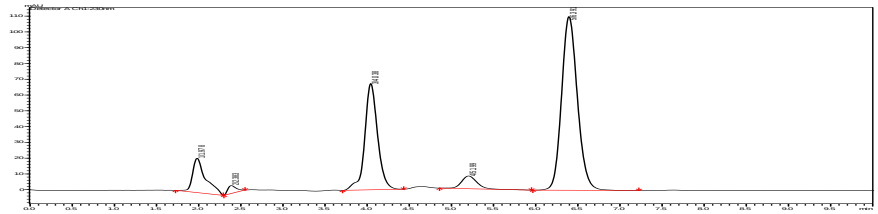

Fig. 7: Calibration studies of 15.25 ppm of REM, VIDA and MET

Table 13: Calibration studies of 15.25 ppm

Peak#	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.
1	1.978	234557	21729	9.2866	1091.892	--	0	1.715
2	2.383	35600	5024	1.4095	2370.743	1.863	0.205	1.526
3	4.038	713758	67285	28.2594	3481.194	7.051	1.042	0.975
4	5.199	102954	8075	4.0762	3868.978	3.816	1.629	1.182
5	6.391	1438872	109972	56.9683	5412.702	3.496	2.232	1.165

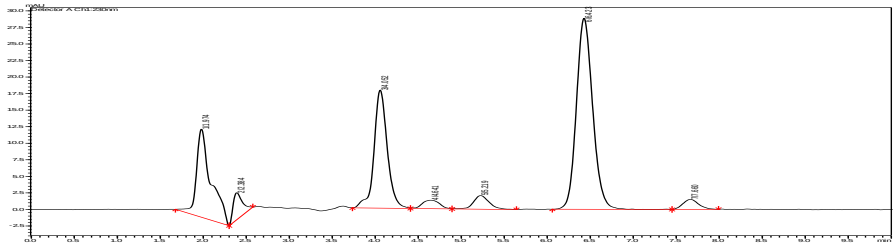

Fig. 8: Calibration studies of 7.5 ppm of REM, VIDA and MET

Table 14: Calibration studies of 7.5 ppm

Peak#	Ret. Time	Area	Height	Area%	T. Plate#	Resolution	k'	Tailing F.
1	1.974	156464	13292	19.0622	1073.192	--	0	1.467
2	2.384	30928	4112	3.768	2194.137	1.847	0.208	1.674
3	4.052	190592	17844	23.22	3528.446	7.001	1.053	0.979
4	4.641	17782	1278	2.1664	2816.277	1.89	1.351	--
5	5.219	26304	2055	3.2046	3841.607	1.684	1.644	1.176
6	6.423	379477	28857	46.2321	5360.766	3.501	2.254	1.159
7	7.66	19262	1509	2.3467	8083.248	3.577	2.881	1.186

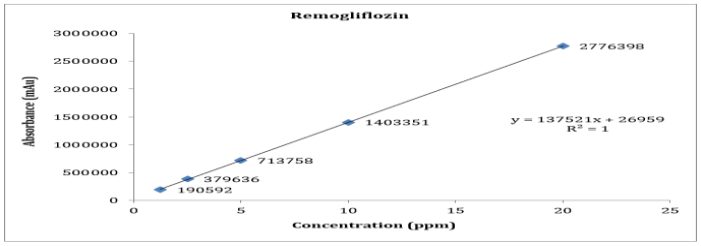

Fig. 9: Calibration curve of Remogliflozin

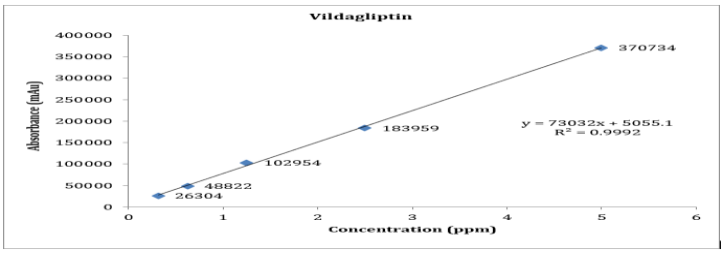

Fig. 10: Calibration curve of Vildagliptin

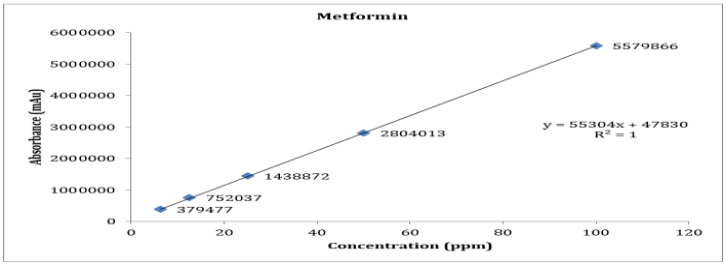

Fig. 11: Calibration curve of Metformin

Table 15: Linearity data of Remogliflozin

Name of Drug:Remogliflozin		
S. No.	**Concentration (µg.mL^{-1})**	**Area**
1	20	2776398
2	10	1403351
3	5	713758
4	2.5	379636
5	1.25	190592
Regression Equation		y = 137521x + 26959
Correlation coefficient (R^2)		1
Std. error of intercept		4789.20779
Std. Dev. Of intercept		10708.99418
LOQ		0.78 µg/ml

LOD	0.23 µg/ml

Table 16: Linearity data of Vildagliptin

Name of Drug:Vildagliptin		
S. No.	**Concentration (µg.mL^{-1})**	**Area**
1	5	370734
2	2.5	183959
3	1.25	102954
4	0.75	48822
5	0.37	26304
Regression Equation		y = 73032x + 5055.1
Correlation coefficient (R^2)		0.9992
Std. error of intercept		3113.822334
Std. Dev. Of intercept		7627.275868
LOQ		0.43 µg/ml
LOD		0.13 µg/ml

Table 17: Linearity data of Metformin

Name of Drug: Metformin		
S. No.	**Concentration (µg.mL^{-1})**	**Area**
1	100	5579866
2	50	2804013
3	25	1438872
4	12.5	752037
5	6.25	379477
Regression Equation		y = 55304x + 47830
Correlation coefficient (R^2)		1

Std. error of intercept	8895.354744
Std. Dev. Of intercept	21789.0802
LOQ	1.61 µg/ml
LOD	0.48 µg/ml

Robustness for the chromatographic method: Robustness of any HPLC method represents its ability to remain unaffected by small but deliberate changes in certain separation factors to ascertain its reliability during routine HPLC analysis. The variation in separation factors such as effect of temperature, flow rate, wavelength, column length, stationary phase particle size, pH, organic modifier composition in mobile phase and injection volume have been considered. The effects of all these variables over changes in retention pattern including effects on capacity/retention factor (k'), resolution (Rs), tailing factor (*Tf*), separation factor, theoretical plates (N) and peak area can be monitored.

In this method, robustness studies were established by making deliberate changes in flow rate (1.0 ± 0.1 ml/minutes), organic modifier as acetonitrile (75±2% ml), and wavelength (210 ±2nm). As shown in results Fig. 12-17; variation in flow rate and organic modifier have made slight changes in retention pattern like increase in flow rate and organic modifier have reduce the retention time, retention factor and resolution whereas decreasing the same variables have marginally extended the retention time, capacity/retention factor (k'), resolution (Rs). As noted, these variations have not made any significant changes in theoretical plates and tailing factor of all selected drugs. Therefore, as displayed in all figures (12-17) and tables (18-26) the robustness studies for simultaneous estimation of Remogliflozin, Vildagliptin and Metformin were almost unchanged which clearly depicts that the proposed HPLC method obliged all minimum requirements led by the ICH guidelines.

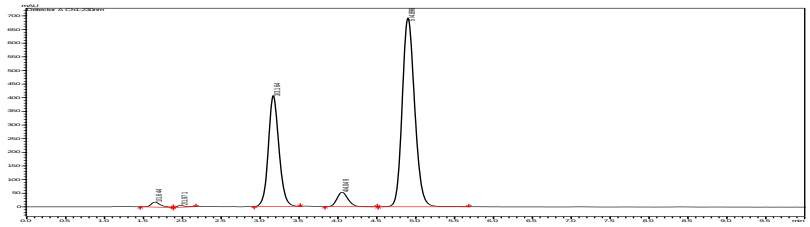

Fig. 12: Effect of flow rate 1.1 ml/min on REM, VIDA and MET

Table 18:Robustness studies, effect of flow rate 1.1 ml/min

Peak#	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.
1	1.644	151277	18092	1.2321	885.499	--	0	1.455
2	1.971	43244	6429	0.3522	1922.323	1.631	0.199	1.728
3	3.164	3848261	405655	31.3415	2446.837	5.477	0.925	1.144
4	4.049	526843	53101	4.2908	3615.224	3.367	1.462	1.202
5	4.896	7708842	692296	62.7834	4296.881	2.984	1.978	1.187

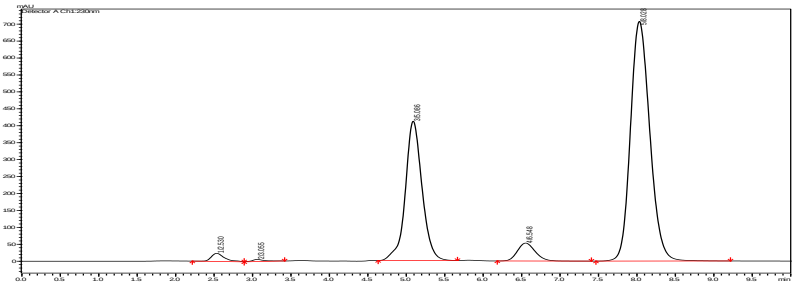

Fig. 13: Effect of flow rate 0.9 ml/min on REM, VIDA and MET

Table 19:Robustness studies, effect of flow rate 0.9 ml/min

Peak#	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.
1	2.53	284120	23870	1.4357	1143.558	--	0	1.579
2	3.055	68088	6208	0.3441	2124.637	1.862	0.208	1.647
3	5.086	6206012	411322	31.3605	2755.32	6.221	1.01	1.009
4	6.548	848840	52961	4.2894	3755.931	3.59	1.588	1.187
5	8.028	12382193	707638	62.5703	4814.898	3.325	2.173	1.179

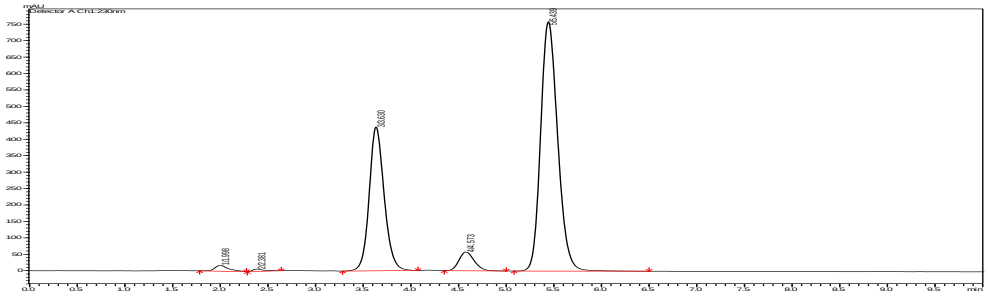

Fig. 14: Effect of organic modifier, solvent B composition %on REM, VIDA and MET

Table 20:Robustness studies, effect of solvent B composition

Peak#	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.
1	1.998	175517	17893	1.1629	1084.075	--	0	1.44
2	2.381	55813	7071	0.3698	2205.04	1.721	0.192	1.867
3	3.63	4785571	437938	31.707	2491.996	5.059	0.817	1.164
4	4.573	639760	57470	4.2388	3670.855	3.181	1.289	1.221
5	5.439	9436430	758374	62.5215	4300.471	2.732	1.722	1.21

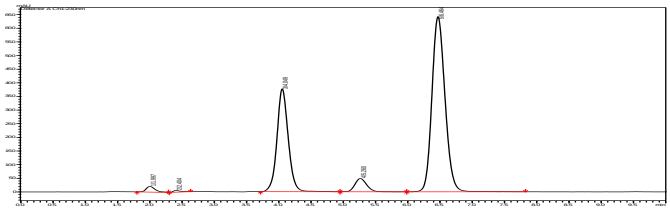

Fig. 15: Effect of organic modifier, solvent B composition %on REM, VIDA and MET

Table 21:Robustness studies, effect of solvent B composition

Peak#	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.
1	1.997	198575	20444	1.3663	989.569	--	0	1.543
2	2.404	49094	6473	0.3378	2294.858	1.788	0.204	1.646
3	4.049	4596240	377042	31.6242	2541.499	6.303	1.027	1.089

4	5.26	616131	48690	4.2393	3815.825	3.66	1.634	1.177
5	6.464	9073903	642042	62.4325	4744.747	3.364	2.237	1.166

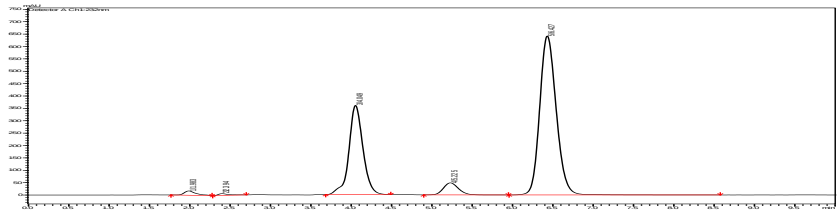

Fig. 16: Effect of wavelength at 232 nm on REM, VIDA and MET

Table 22: Robustness studies, effect of wavelength 232 nm

Peak#	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.
1	1.983	172980	17150	1.2035	976.995	--	0	1.566
2	2.394	60524	6952	0.4211	1995.019	1.759	0.208	2.053
3	4.049	4355617	359836	30.3043	2706.049	6.295	1.042	0.983
4	5.225	631573	48587	4.3942	3627.766	3.571	1.635	1.186
5	6.427	9152221	642366	63.6769	4645.491	3.32	2.242	1.182

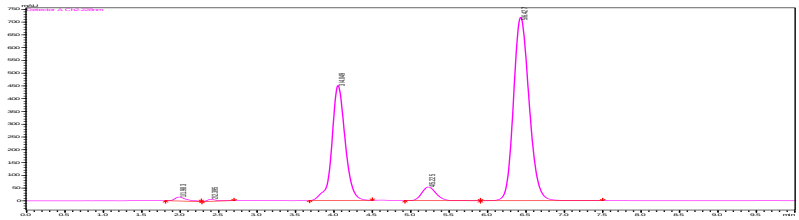

Fig. 17: Effect of wavelength at 228 nm on REM, VIDA and MET

Table 23:Robustness studies, effect of wavelength 228 nm

Peak#	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.
1	1.983	157713	15576	0.9564	969.424	--	0	1.62
2	2.395	62045	7094	0.3763	2001.375	1.756	0.208	2.05
3	4.049	5389574	449089	32.6851	2730.955	6.316	1.042	1
4	5.225	696304	53571	4.2227	3635.399	3.58	1.635	1.186
5	6.427	10183769	718273	61.7595	4677.243	3.327	2.241	1.184

Table 24:Robustness data of Remogliflozin

Variables	Remogliflozin			
	tR(min)	k'	Tf	N
Flowrate(+0.2mL.min^{-1})	3.16	0.92	1.14	2446
Flowrate(-0.2mL.min^{-1})	5.08	1.01	1	2755
CH3OH(+2%)	3.63	0.81	1.16	2491
CH3OH(-2%)	4.04	1.02	1.08	2541
Temperature(+2˚C)	4.04	1.04	0.98	2706

Temperature(-2˚C)	4.04	1.04	1.01	2730
Mean±S.D.	4.00 ± 0.63	0.97 ± 0.09	1.06 ± 0.08	

Table 25:Robustness data of Vildagliptin

Variables	Vildagliptin				
	tR(min)	**k'**	**Tf**	**Rs**	**N**
Flowrate(+0.2mL.min^{-1})	4.04	1.46	1.2	3.36	3615
Flowrate(-0.2mL.min^{-1})	6.54	1.58	1.18	3.59	3755
CH3OH(+2%)	4.57	1.28	1.22	3.18	3670
CH3OH(-2%)	5.26	1.63	1.17	3.58	3815
Temperature(+2˚C)	5.22	1.63	1.18	3.57	3627
Temperature(-2˚C)	5.22	1.63	1.18	3.58	3635
Mean±S.D.	5.14 ± 0.84	1.54 ± 0.14	1.19 ± 0.02	3.48±0.17	

Table 26:Robustness data of Metformin

Variables	Metformin				
	tR(min)	**k'**	**Tf**	**Rs**	**N**
Flowrate(+0.2mL.min^{-1})	4.89	1.97	1.18	2.98	4216
Flowrate(-0.2mL.min^{-1})	8.08	2.17	1.17	3.32	4814
CH3OH(+2%)	5.43	1.72	1.21	2.73	4300
CH3OH(-2%)	6.46	1.16	1.09	3.58	4744
Temperature(+2˚C)	6.42	2.24	1.18	3.32	4645
Temperature(-2˚C)	6.42	2.24	1.18	3.32	4677
Mean±S.D.	6.28 ± 1.09	1.92 ± 0.42	1.17 ± 0.04	3.21 ± 0.30	

Accuracy studies of Marketed Formulation: Percentage drug accuracy of three different concentrations; 80%, 100% and 120% (injected thrice) to estimate the Vildagliptin and Metformin from marketed formulation and results obtained have been reported in **Table 27-30.**Accuracy can be studied by applying the calibration curve, the Y-intercept and the slope of the graph were used to determine the % drug recovery, attributed to the developed method for the simultaneous quantification of selected drugs or by comparing with similar concentration of reference standard. As resulted, the achieved drug recovery of both Vildagliptin and Metformin were in the range of 100.4-100.7 and 100-105, respectively. As recommended by international conferences of Harmonization (ICH) guidelines the drug recovery should be within the range of 90-110% and the RSD in percentage should be less than 2%. Hence, the calculated drug recoveries for simultaneous estimation of Vildagliptin and Metformin represents the drug recovery were in the acceptance limit given by ICH guidelines.

Table 27: Marketed formulation

Peak#	Ret. Time	Area	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation

1	1.988	192324	1.3518	1028.858	--	0	1.464	0
2	2.383	52123	0.3664	2254.602	1.76	0.199	1.9	0
Remogliflozin	3.816	4451020	31.2849	2501.737	5.664	0.919	1.12	4.629
Vildagliptin	4.874	603526	4.242	3654.78	3.371	1.451	1.197	1.579
Metformin	5.888	8928364	62.7549	4451.658	3.001	1.961	1.185	1.351

Table 28: Accuracy data of Vildagliptin

Drug Name: Remogliflozin			**Drug content: 50 mg**		**Marketed formulation: Remo MV 500 Tablet**		
Std. conc. (%)	**Std. (ppm)**	**Peak area**	**Drug (%)**	**Drug (ppm)**	**Peak area**	**Avg. peak area**	**Drug Rec. (%)**
100%	5 ppm	600834	80	4	482821	488131	101.55
				4	493441		
			100	5	603526	613932	102.18
				5	624338		
			120	6	724231	722127	100.16
				6	720023		
			Drug recovery Range (%) as per ICH = 100±10%				100.16 – 102.18 %

Table29: Accuracy data of Remogliflozin

Drug Name: Remogliflozin etabonate			**Drug content: 100 mg**		**Marketed formulation; Remo MV 500 Tablet**		
Std. conc. (%)	**Std. (ppm)**	**Peak area**	**Drug (%)**	**Drug (ppm)**	**Peak area**	**Avg. peak area**	**Drug Rec. (%)**
100%	20 ppm	4537971	80	16	3560816	3514965	96.82
				16	3469114		
			100	20	4451020	4518725	99.58
				20	4586429		
			120	24	5341224	5362623	98.48
				24	5384021		
			Drug recovery Range (%) as per ICH = 100±10%				96.82 – 99.58 %

Table 30: Accuracy data of Metformin

Drug Name: Metformin			**Drug content: 500 mg**		**Marketed formulation; Remo MV 500 Tablet**		
Std. conc. (%)	**Std. (ppm)**	**Peak area**	**Drug (%)**	**Drug (ppm)**	**Peak area**	**Avg. peak area**	**Drug Rec. (%)**
100%	100 ppm	8964174	80	80	7142691	7082173	98.76
				80	7021655		
			100	100	8928364	9061489.5	101.09
				100	9194615		
			120	120	10714037	10367834	96.38
				120	10021632		
			Drug recovery Range (%) as per ICH = 100±10%				96.38 – 101.09 %

Conclusion: As Metformin is too polar to be retain by Reverse phase mode on C18 column. The present HPLC Method by using Acclaimed mixed mode column exhibit simultaneous estimation of highly polar Metformin with Remogliflozin and Vidagliptin with proper resolution and selectivity as compared to C18 column where all the analytes were eluted quite late with void volume and peaks were overlapped results of repeatability, linearity, accuracy, precision, and robustness and specificity were found acceptable and validated as per the ICH guidelines. The established method was found stable since there was no interference of degradants in force degradation was observed.

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