



DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF REMOGLIFLOZIN AND VILDAGLIPTIN IN PHARMACEUTICAL DOSAGE FORM

Submitted By

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ABSTRACT

The present study was focused to develop a simple, precise, accurate and cost effective RP-HPLC method for estimation of Remogliflozin etabonate and Vildagliptin from pharmaceutical dosage form. The chromatographic method was carried out using Agilent C₁₈ (150mm*4.6mm) 5 μ m using mobile phase (Methanol: Buffer (pH3.5): ACN (55:35:10 %v/v)). The flow rate was set 1.0ml/min with 20 μ L injection volume. Total run time 10min. Detection was carried out at wavelength of 254nm. The detector response was linear in the range of 150-450 μ g/ml for Remogliflozin etabonate and 75-225 μ g/ml for Vildagliptin. The % recoveries for Remogliflozin etabonate and Vildagliptin obtained in the accuracy study were 99.07-100.38% and 99.69-100.70% respectively. The LOD for Remogliflozin etabonate and Vildagliptin were found to be 0.0053 μ g/ml and 0.0038 μ g/ml respectively. LOQ for Remogliflozin etabonate and Vildagliptin were found to be 0.016 μ g/ml and 0.0011 μ g/ml respectively. Remogliflozin etabonate and Vildagliptin were also subjected to various stress condition like acid and alkali hydrolysis, oxidation, photolysis, and thermal degradation. The developed method is successfully applied for estimation of Remogliflozin etabonate and Vildagliptin from pharmaceutical dosage form.

Keywords: Remogliflozin etabonate, RP-HPLC, Method Development, Stability Study, Validation

1. INTRODUCTION

1.1 INTRODUCTION TO DIABETES MELLITUS:- ^[1-2]

Definition:

“Diabetes mellitus (DM), commonly referred to as diabetes, is a group of metabolic diseases in which there are high blood sugar levels over a prolonged period.”

Description:

Diabetes is due to either the pancreas not producing enough insulin or the cells of the body not responding properly to the insulin produced.

There are three main types of diabetes mellitus:

Type 1 Diabetes mellitus:

Type 1 Diabetes mellitus results from the pancreas's failure to produce enough insulin. This form was previously referred to as "insulin-dependent diabetes mellitus" (IDDM) or "juvenile diabetes". The cause is unknown.

Type 2 Diabetes mellitus:

Type 2 Diabetes mellitus begins with insulin resistance, a condition in which cells fail to respond to insulin properly. As the disease progresses a lack of insulin may also develop. This form was previously referred to as "non-insulin-dependent diabetes mellitus" (NIDDM) or "adult-onset diabetes". The primary cause is excessive body weight and not enough exercise.

Gestational diabetes:

Gestational diabetes is the third main form and occurs when pregnant women without a previous history of diabetes develop high blood-sugar levels.

1.1.1 PATHOPHYSIOLOGY OF DIABETES:- ^[1-2]

Pharmacology of Diabetes:

It is type of endocrine disorder which is characterized by

1. Thickening of capillary membranes throughout the body
2. Extensive disturbances of carbohydrate, protein and lipid metabolism which in turn cause Deranged glucose secretion.

3. Long term complication involving various organs like eye (cataract), kidney (renal failure), peripheral nervous system (neuropathy).

Table 1 Differences between Type-1 and Type-2 Diabetes

Characteristics	Type-1 Diabetes	Type-2 Diabetes
Etiology	Autoimmune	Peripheral insulin resistance
Formerly known as	IDDM or “juvenile”	NIDDM or “Adult Onset”
Age of onset	Younger	Older
Ketosis	Yes	No
Presence of body’s Own insulin	No	Yes
Obesity	Rare	Common
Family history	Rare	Common
Insulin resistance	No	Yes
Response to oral agents	No	Yes

Symptoms:

Symptoms of high blood sugar include polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger). If left untreated, diabetes can cause many complications. Acute complications can include diabetic ketoacidosis, nonketotic hyperosmolar coma, or death. Serious long-term complications include heart disease, stroke, chronic kidney failure, foot ulcers, slow healing of cuts, itchy skin and damage to the eyes. Symptoms may develop rapidly (weeks or months) in type 1 DM, while they usually develop much more slowly and may be subtle or absent in type 2 DM.

Prevention and Treatment:

Prevention and treatment involve maintaining a healthy diet, regular physical exercise, a normal body weight, and avoiding use of tobacco. Control of blood pressure and maintaining proper foot care are important for people with the disease.

Type 1 DM must be managed with insulin injections.

Type 2 DM may be treated with medications with or without insulin. Insulin and some oral medications can cause low blood sugar, Weight loss surgery in those with obesity is sometimes an effective measure in those with type 2 DM.

Gestational diabetes usually resolves after the birth of the baby

1.1.2 ANTIDIABETIC AGENTS:- [1-2]

Antidiabetic agents aim to achieve normoglycemia and relieve diabetes symptoms, such as thirst, polyuria, weight loss and ketoacidosis. The long term goals are to prevent the development of or slow the progression of long term complications of the disease. Choice of Antidiabetic agent depends on the type of diabetes.

Type I diabetes is where the body does not produce any insulin, so insulin is the only treatment choice. Injected insulin acts similar to endogenous insulin to lower blood glucose levels.

Type 2 Diabetes is first treated with oral Antidiabetic medicines. These medicines, make the pancreas produce more insulin, help decrease insulin requirements by the body or reduces gluconeogenesis by the liver. If normoglycemia is not achieved with oral medicines then insulin can be added to the therapy.

Classification of Antidiabetic agents

Biguanides: Metformin

Sulfonylureas: Glyburide, Glimepiride, Glipizide

Meglitinide derivatives: Repaglinide, Nateglinide

Alpha-glucosidase inhibitors: Acarbose, Voglibose, Miglitol

Thiazolidinediones: Pioglitazone, Rosiglitazone

Glucagon like peptide–1 (GLP-1) agonists: Exenatide, Liraglutide, Albiglutide

Dipeptidyl peptidase IV (DPP-4) Inhibitors: Linagliptin, Sitagliptin, Saxagliptin, Vildagliptin

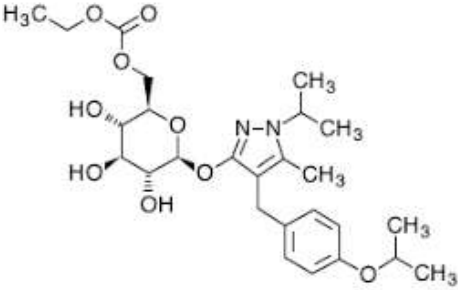
Selective sodium-glucose transporter-2 (SGLT-2) inhibitors: Empagliflozin, Canagliflozin, Remogliflozin etabonate

Insulins: Insulin aspart, Insulin lispro, Insulin detemir

Amylinomimetics: Pramlintide

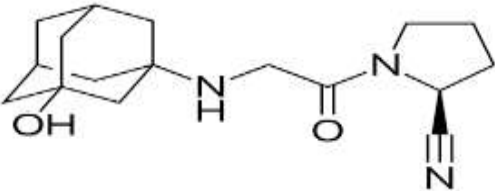
Bile acid sequestrants: Colesevelam

2 DRUG PROFILE:-**2.1 Remogliflozin etabonate:-** [21-24]

INTRODUCTION	
Name	Remogliflozin etabonate
Official in	Not Official in any Pharmacopoeia
Description	Remogliflozin is a sodium glucose co-transporter-2 (SGLT-2) inhibitor indicated as an adjunct to diet and exercise to improve glycemic control in adult patients with type 2 diabetes. SGLT2 co-transporters are responsible for reabsorption of glucose from the glomerular filtrate in the kidney. The glucuretic effect resulting from SGLT2 inhibition reduces renal absorption and lowers the renal threshold for glucose, therefore resulting in increased glucose excretion.
Structure	
Chemical Formula	C ₂₆ H ₃₈ N ₂ O ₉
Mol. Weight	522.6 g/mol
IUPAC Name	(ethyl (((2R,3S,4S,5R,6S)-3,4,5-trihydroxy-6-((4-(4-isopropoxybenzyl)-1-isopropyl-5-methyl-1H-pyrazol-3-yl)oxy)tetrahydro-2H-pyran-2-yl)methyl) carbonate
Categories	Antidiabetes drug
Solubility	Soluble in methanol and DMSO (di methyl sulfoxide)

PHARMACOLOGY									
Classes	SGLT-2 Inhibitors								
Mechanism of action	Remogliflozin etabonate is a sodium glucose co-transporter-2(SGLT-2) inhibitor. SGLT2 co-transporters are responsible for reabsorption of glucose from the glomerular filtrate in the kidney. The glucuretic effect resulting from SGLT2 inhibition reduces renal absorption and lowers the renal threshold for glucose, resulting in increased glucose excretion. Additionally, it contributes to reduced hyperglycemia, assists weight loss, and reduces blood pressure.								
Indication	To treat type 2 Diabetes mellitus								
Half Life	120 min								
PROPERTIES									
State	White crystalline Solid								
CAS NO.	442201-24-3								
Melting Point	95 ⁰ C-105 ⁰ C								
Storage	Store between 15 ⁰ C- 30 ⁰ C								
Experimental properties	<table border="1"> <thead> <tr> <th>Property</th> <th>Value</th> </tr> </thead> <tbody> <tr> <td>Water solubility</td> <td>Insoluble</td> </tr> <tr> <td>Log P</td> <td>2.50</td> </tr> <tr> <td>pKa</td> <td>12.58</td> </tr> </tbody> </table>	Property	Value	Water solubility	Insoluble	Log P	2.50	pKa	12.58
Property	Value								
Water solubility	Insoluble								
Log P	2.50								
pKa	12.58								

2.2 VILDAGLIPTIN:- [25-26]

INTRODUCTION	
Name	VILDAGLIPTIN
Official in	Not Official in any Pharmacopoeia
Description	Vildagliptin is a potent, orally bio available dihydropurinedione-based inhibitor of dipeptidyl peptidase 4 (DPP-4). It is used as a hypoglycemic agent in the treatment of TYPE II Diabetes Mellitus.
Structure	
Chemical Formula	$C_{17}H_{25}N_3O_2$
Mol. Weight	303.4 g/mol
IUPAC Name	(2s)-1-[2-[3-hydroxy-1-adamantyl)amino] acetyl] pyrrolidine-2-carbonitrile
Categories	Antidiabetic drug
Solubility	Soluble in methanol; sparingly soluble in water
PHARMACOLOGY	
Classes	DPP-4 inhibitor

Mechanism of action	Vildagliptin is a competitive and reversible dipeptidyl peptidase (DPP)-4 enzyme inhibitor that slows the breakdown of insulinotropic hormone glucagon-like peptide (GLP)-1 for better glycemic control in diabetes patients. GLP and glucose-dependent insulinotropic polypeptide (GIP) are incretin hormones that increase the production and release of insulin from pancreatic beta cells and decrease the release of glucagon from pancreatic alpha cells. This results in an overall decrease in glucose production in the liver and increase in insulin in a glucose-dependent manner									
Indication	To treat type 2 Diabetes mellitus									
Half Life	90 min									
PROPERTIES										
State	White to Yellow crystalline solid									
CAS NO.	668270-12-0									
Melting point	149 ⁰ C-155 ⁰ C									
Storage	Store between 8 ⁰ C-25 ⁰ C									
Experimental Properties	<table border="1"> <thead> <tr> <th>Property</th> <th>Value</th> </tr> </thead> <tbody> <tr> <td>Water solubility</td> <td>Freely soluble in water mg/ml</td> </tr> <tr> <td>Log P</td> <td>1.12</td> </tr> <tr> <td>pKa</td> <td>9.03</td> </tr> </tbody> </table>		Property	Value	Water solubility	Freely soluble in water mg/ml	Log P	1.12	pKa	9.03
Property	Value									
Water solubility	Freely soluble in water mg/ml									
Log P	1.12									
pKa	9.03									

3. EXPERIMENTAL WORK

3.1 DRUG IDENTIFICATION :-

The identification of standard API for experimental work was done for confirmation of its identity, standard, quality and purity. The identification was done by taking IR and UV spectra, solubility study and Melting point determination.

UV absorption and selection of Detection Wavelength

50 mg of Remogliflozin etabonate and 25 mg Vildagliptin were taken and transferred to 50 ml volumetric flask separately that is dissolved in methanol. Make further dilution by taking 3ml from the stock solution of Remogliflozin etabonate and Vildagliptin separately in to 10 ml volumetric flask. A UV Spectra was taken between 200-400nm using UV-Visible double beam spectrometer. Remogliflozin etabonate and Vildagliptin both drug give appreciable absorbance at 254nm. So 254 nm has been selected UV Overlain Spectra is shown in Figure

3.2 METHOD DEVELOPMENT:-

Selection of Column

Inertsil C₁₈ (250mm*4.6mm) 5µm, thermofisher C₁₈(150mm*4.6mm) 5µm , Agilent C₁₈ (150mm*4.6mm) 5µm , were used for method development. Finally Agilent C₁₈ (150mm*4.6mm) 5µm was used for better resolution of peak of analyte.

Selection of Mobile Phase

Mobile phase selection involved selection of buffer, pH of buffer, selection of solvents and ratio of buffer and solvent. The standard solutions of Carvedilol and Ivabradinewere injected into the HPLC system and run in different solvent system. Various ratios of mobile phase containing Methanol:Water, Methanol:Phosphate buffer, Methanol:ACN,Methanol:Water:ACN, Methanol:ACN:Buffer were tried in order to find the best conditions for the separation of both drugs. It was found that Methanol , Phosphate buffer with pH 3.5 adjusted by 1% Ortho phosphoricacid and ACN gives satisfactory result.

Finally, Methanol: Buffer: ACN(55:35:10 %v/v) ratio was optimized as the mobile phase for the determination.

3.2.1 Preparation of Solutions

Preparation of Diluent

Based in literature review and based on solubility on Remogliflozin etabonate and Vildagliptin, drugs were soluble in Methanol but Methanol peak shape was not good.So use Methanol: 0.1M Potassium Dihydrogen Phosphate (70:30) as Diluents. Prepare 0.1M Potassium Dihydrogen Phosphate by dissolving 13.6 gm in 1000ml of water. Adjust pH 3.5 with OPA solution. This solution was sonicated for 5 min degassing.

Preparation of the Buffer solution

Transferred accurately 13.6gm Potassium Dihydrogen Phosphate into 1000ml of water , Dissolve and filter it. And pH adjusted 3.5 with 1% ortho phosphoric acid .

(preparation of 1% ortho phosphoric acid- 1ml of ortho phosphoric acid into 1000ml of water)

Preparation of Mobile phase

Prepare 0.1M Potassium Dihydrogen Phosphate by dissolving 13.6 gm of Potassium Dihydrogen Phosphate in 1000ml of water. Adjust pH 3.5 with OPA solution. This solution was sonicated for 5 min for degassing and filtered through 0.45 μ Millipore filter. Prepare the ratio of Methanol:0.1M Phosphate Buffer:ACN(55:35:10)% v/v.

3.2.2 Preparation of Standard Solutions

- **Preparation of Standard solution of Remogliflozin etabonate**

Accurately weighed quantity of Remogliflozin etabonate (50mg) was transferred into 50ml volumetric flask, sonicated to dissolve and diluted up to mark with diluent to give a stock solution. An aliquot (3.0 ml) of the solution was transferred to 10 ml volumetric flask and diluted to the mark with diluent to obtain a working standard solution of Remogliflozin etabonate.

- **Preparation of Standard Solution of Vildagliptin**

Accurately weighed quantity of Vildagliptin (25.00 mg) was transferred into 50ml volumetric flask, sonicated to dissolve and diluted up to mark with diluent to give a stock solution. An aliquot (3.0 ml) of the solution was transferred to a 10 ml volumetric flask and diluted to the mark with diluent to obtain a working standard solution of Vildagliptin.

3.2.2.1 Preparation of Sample Solution of Remogliflozin etabonate and Vildagliptin

(Label claim: Remogliflozin etabonate-100mg; Vildagliptin-50mg) Twenty tablets were weighed; calculate the average and finely powdered tablets. Powder is added into 50ml of volumetric flask and add 25 ml of Diluent and it sonicated for 5 min with shaking of 1 min interval. 25ml of Diluent is added into this flask and further sonicated for 10min with shaking of 1min interval. Filtered the solution through 0.45 μ m filter paper.

Final chromatographic condition

Buffer	13.6gm Potassium Dihydrogen Phosphate into 1000ml of water, And pH adjusted 3.5 with 1% ortho phosphoric acid .
Mobile phase	Methanol: Buffer: ACN (55:35:10 %v/v)
Column	Agilent C ₁₈ (150mm*4.6mm) 5 μ m
Wavelength	254nm
Flow rate	1.0 ml/min
Injection volume	20 μ l

Run time	10 min
Column temperature	30°C

4. FORCED DEGRADATION:

Preparation of reagents:

- **0.1N HCl:** 8.5 mL Conc. Hydrochloric acid was taken in 100 mL volumetric flask and Volume was made upto the mark with water and mix well.
- **0.1N NaOH:** 4 gm NaOH pellets was taken in 100 mL volumetric flask and Volume was made upto the mark with water and mix well.
- **3 % H₂O₂:** 10.0 mL from 30 % H₂O₂ solution was taken in 100 mL volumetric flask, Volume was made up to mark with water and mix well.

4.1 Acid Degradation:-

Weighed accurately 20 tablets and calculate the average weight. Tablets were crushed into fine powder and mix well. Weighed and transferred crushed tablet powder equivalent to 50 mg of Remoglifloglin etabonate and 25mg Vildagliptin transferred into 50 mL volumetric flask. Accurately 30 mL of diluent (Methanol:Buffer (70 : 30) % v/v) was added and sonicated for 5 min to ensure complete solubilization of drug. After sonication, volume was made up to the mark with diluent(stock solution). The solution was filtered through 0.45µ Milipore filter. From this stock solution 3ml of solution was pipetted out in 10 ml volumetric flask and volume was made up to the mark with diluent. (300ppm Remogliflozin etabonate and 150 ppm Vildagliptin).Pipette out 3ml from 300ppm Remogliflozin etabonate and 150ppm Vildagliptin,1ml of 0.1N hydrochloric acid was added and keep it at room temperature for 1 hours. After that it was neutralized by adding 1ml of 0.1N NaOH & volume was made up to 10ml diluent.For analysis of standard solution 300ppm Remogliflozin etabonate and 150ppm also kept under acid degradation same as sample solution. After making final dilution the standard and sample solution were injected into HPLC and peak area and peak shape were observed. Chromatogram are shown in Figure

4.2 Base degradation:-

Weighed accurately 20 tablets and calculate the average weight. Tablets were crushed into fine powder and mix well. Weighed and transferred crushed tablet powder equivalent to 50 mg of Remoglifloglin etabonate and 25mg Vildagliptin transferred into 50 mL volumetric flask. Accurately 30 mL of diluent (Methanol:Buffer (70 : 30) %

v/v) was added and sonicated for 5 min to ensure complete solubilization of drug. After sonication, volume was made up to the mark with diluent(stock solution). The solution was filtered through 0.45 μ Milipore filter. From this stock solution 3ml of solution was pipetted out in 10 ml volumetric flask and volume was made up to the mark with diluent. (300ppm Remogliflozin etabonate and 150 ppm Vildagliptin).Pipette out 3ml from 300ppm Remogliflozin etabonate and 150ppm Vildagliptin,1ml of 0.1N NaOH was added and keep it at room temperature for 1 hours. After that it was neutralized by adding 1ml of 0.1N Hydrochloric acid & volume was made up to 10ml diluent.For analysis of standard solution 300ppm Remogliflozin etabonate and 150ppm Vildagliptin also kept under base degradation same as sample solution. After making final dilution the standard and sample solution were injected into HPLC and peak area and peak shape were observed. Chromatogram are shown in Figure

4.3 Peroxide degradation:-

Weighed accurately 20 tablets and calculate the average weight. Tablets were crushed into fine powder and mix well. Weighed and transferred crushed tablet powder equivalent to 50 mg of Remogliflozin etabonate and 25mg Vildagliptin transferred into 50 mL volumetric flask. Accurately 30 mL of diluent (Methanol:Buffer (70 : 30) % v/v) was added and sonicated for 5 min to ensure complete solubilization of drug. After sonication, volume was made up to the mark with diluent(stock solution). The solution was filtered through 0.45 μ Milipore filter. From this stock solution 3ml of solution was pipetted out in 10 ml volumetric flask and volume was made up to the mark with diluent. (300ppm Remogliflozin etabonate and 150 ppm Vildagliptin).Pipette out 3ml from 300ppm Remogliflozin etabonate and 150ppm Vildagliptin,1ml of 3% H_2O_2 was added and keep it at room temperature for 1 hours. After that volume was made up to 10ml with diluent.For analysis of standard solution 300ppm Remogliflozin etabonate and 150ppm also kept under peroxide degradation same as sample solution. After making final dilution the standard and sample solution were injected into HPLC and peak area and peak shape were observed. Chromatogram are shown in Figure

4.4 Thermal Degradation:-

Twenty tablets were weighed, Powdered. Tablet powder equivalent to 50 mg of Remogliflozin etabonate and 25 mg Vildagliptin was taken. It was kept in hot air oven at 60 $^{\circ}C$ for 2 hours.After that powder transferred to 50 ml volumetric flask and volume was made up to 50 ml with diluents. An aliquot (3ml) was diuted up to 10ml with diluent (300ppm Remogliflozin etabonate and 150 mg Vildagliptin). Filter the final solution with 0.45 μ PVDF filter. Pipette out 3ml from 300ppm Remogliflozin etabonate and Vildagliptin in 10ml volumetric flask and volume was made up with diluent. For analysis of standard 50mg Remogliflozin etabonate and 25mg Vildagliptin taken and kept in hot air oven at 60 $^{\circ}C$ for 1hours. After that dilutions were made to make final solutions of 300ppm Remogliflozin etabonate and 150ppm Vildagliptin. Solution were injected in HPLC and the peak area and peak shape were observed. Chromatogram are shown in Figure

4.5 Photo Degradation:-

Twenty tablets were weighed, powdered. Tablet powder equivalent to 50mg of Remogliflozin etabonate and 25mg Vildagliptin was taken. It was exposed to direct sun light for 1hours. After that powder transferred to the 50ml volumetric flask and volume was made up to 50ml with diluent. An aliquot(3ml) was diluted up to 10ml with diluent (300ppm Remogliflozin etabonate and 150 Vildagliptin).Filter the final solution with 0.45 μ PVDF filter. Pipette out 3ml from 300ppm Remogliflozin etabonate and 150ppm Vildagliptin in 10ml volumetric flask and volume was made u with diluent. For analysis of Standard 50mg of Remogliflozin etabonate and Vildagliptin taken and exposed to sun light for 1 hours. After that dilutions were made to make final solutions of 300ppm Remogliflozin etabonate and 150ppm Vildagliptin. Solutions were injected in HPLC and the peak area and peak shape were observed. Chromatogram are shown in figure

6.Validation:-

Preparation of solutions

Diluents:- Based in literature review and based on solubility on Remogliflozin etabonate and Vildagliptin, drugs were soluble in Methanol but Methanol peak shape was not good.So use Methanol: 0.1M Potassium Dihydrogen Phosphate (70:30) as Diluents. Prepare 0.1M Potassium Dihydrogen Phosphate by dissolving 13.6 gm in 1000ml of water. Adjust pH 3.5 with OPA solution. This solution was sonicated for 5 min degassing.

Preparation of standard:- 50 mg of Remogliflozin etabonate and 25 mg Vildagliptin were taken and transferred to 50 ml volumetric flask separately and volume was made up with diluent.3 ml from Remogliflozin etabonate and Vildagliptin stock solution was taken into 10 ml volumetric flask and volume was made up by diluent (300 μ g/ml Remogliflozin etabonate and 150 μ g/ml Vildagliptin).

Preparation of Calibration curve solutions: Aliquots equivalent to 1.5,2.4,3.0,3.6 and 4.5 ml of working standard solution of Remogliflozin etabonate and 1.5,2.4,3.0,3.6, and 4.5 ml of working standard solution were transferred to series of five 10 ml volumetric flasks and volume was adjusted to the mark with diluent to get concentration of 150,240,300,360 and 450 μ g/ml of Remogliflozin etabonate and 75,120,150,180 and 225 μ g/ml. 20 μ l of each of the solutions were injected into HPLC system and analysed. Calibration curve was obtain by plotting respective peak area against concentration in μ g/ml and regression equation was computed.

6.1 System Suitability Test:-

System suitability was performed by preparing solutions as per the test method and analysed before performing any validation parameters to verif that the system is adequate for the analysis. The parameters used to verify in this test were retention time, theoretical plates,tailing factor and resolution. The values of system suitability results obtained are shown in table.

Acceptance criteria

- %RSD of Area of five replicate standard injections should not be more than 2.0
- Theoretical Plates for the analyte peak should not be less than 2000.
- Tailing factor for the analyte peak should not more than 2.0

6.2 Specificity

In the case of assay, demonstration of specificity is required to show that the procedure is unaffected by the presence of impurities or excipients. Specificity of an analytical method indicates that the analytical method is able to measure accurately and specifically the analyte of interest without any interference from blank. So here, the specificity was determined by the comparison of the chromatograms of

- Blank (mobile phase).
- Standard sample solutions of Remogliflozin etabonate and Vildagliptin.
- Sample solution of Remogliflozin etabonate and Vildagliptin.

6.3 Linearity and Range

Linearity response was determined by analysing different concentrations for calibration curve in the range of 150-450 µg/ml for Remogliflozin etabonate and 75-225 µg/ml for Vildagliptin. Peak areas were measured at each level. Peak areas were plotted against concentration and equation of straight line and correlation co-efficient was determined. Chromatogram and curve is shown in Figure & Data of Linearity are shown in table.

Acceptance criteria: value of r^2 should be nearer to 1 or 0.999

6.4 Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision considered at three levels: repeatability, intermediate (intraday) precision and reproducibility (interday) precision.

Intraday precision (n=3)

Solution containing (150,300,450) µg/ml of Remogliflozin etabonate and (75,150,225) µg/ml of Vildagliptin were analysed three times on same day and % RSD was calculated. Data is shown in table.

Interday precision (n=3)

Solutions containing (150,300,450) µg/ml of Remogliflozin etabonate and (75,150,225) µg/ml of Vildagliptin were analysed on three different successive and % RSD was calculated. Data is shown in table.

Repeatability (n=6)

Method precision of experiment was performed by preparing the standard solutions of Remogliflozin etabonate(300 µg/ml) and Vildagliptin (150 µg/ml) for six times and analysed as per proposed method and % RSD was calculated. Data is shown in table

Acceptance criteria: %RSD of area should not be more than 2.0%.

6.5 Accuracy

The accuracy of the method was determined at 50%,100%,150% by calculating recoveries of Remogliflozin etabonate and Vildagliptin by the standard addition method. Known amount of standard solution of Remogliflozin etabonate (150,300,450 µg/ml) and Vildagliptin (75,150,225 µg/ml) were added to a pre-quantified sample solution of Remogliflozin etabonate (300 µg/ml) and Vildagliptin (150 µg/ml). Each solution was injected in triplicate and the percentage recovery was calculated by measuring the peak areas and fitting these values into the regression equation of the respective calibration curves. Data is shown in table.

6.6 Robustness

The robustness was studied by analysing the samples of Remogliflozin etabonate and Vildagliptin by deliberate variation in the method parameters. The Change in the response of Remogliflozin etabonate and Vildagliptin was noted. Robustness of the method was studied by changing flow rate by ± 0.2 ml/min, and Temperature $\pm 2^{\circ}$ C. The changes in the response of Remogliflozin etabonate and Vildagliptin were noted and compared with the original one.

-Flow Rate: 0.8ml/min and 1.2ml/min

-Temperature: 28⁰C and 30⁰C

Acceptance criteria:

Number of theoretical plates for the analyte peak should not be less than 2000.

Asymmetry value for the analyte peak should not be more than 2.0

%RSD for the analyte peak should not be more than 2.0%

Data of Robustness are shown in table.

6.7 Applicability of Method for Marketed Formulation

Twenty tablets were weighed and powdered. Tablet powder equivalent to 50mg of Remogliflozin etabonate and 25mg Vildagliptin was transferred to 50 ml volumetric flask. About 30ml diluent was added and sonicated for 5 min to the mark with diluent(stock solution). The solution was filtered through 0.45µ Milipore filter. From this stock solution 3ml

of solution was pipetted out in 10 ml volumetric flask and volume was made up to the mark with diluent.(300 $\mu\text{g/ml}$ Remogliflozin etabonate and 150 $\mu\text{g/ml}$ Vildagliptin). The quantification was carried out by keeping these values to be straight line equation of calibration curve. Chromatogram shown in figure & Data of analysis of marketed formulation are shown in table.

7 RESULT AND DISCUSSION

7.1 UV Absorption Spectra and Determination of Detection Wavelength

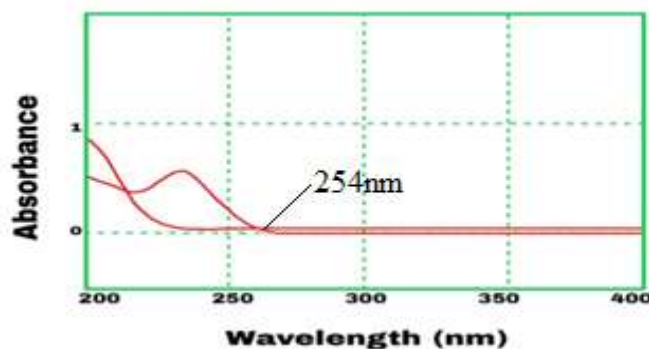


Figure 1 UV Overlain Spectra of Remogliflozin etabonate and Vildagliptin

Observation:-Remogliflozin etabonate and Vildagliptin both drug give significance absorbance at 254 nm. So 254nm has been selected as detection Wavelength.

(Final Chromatographic Condition for Optimized method)

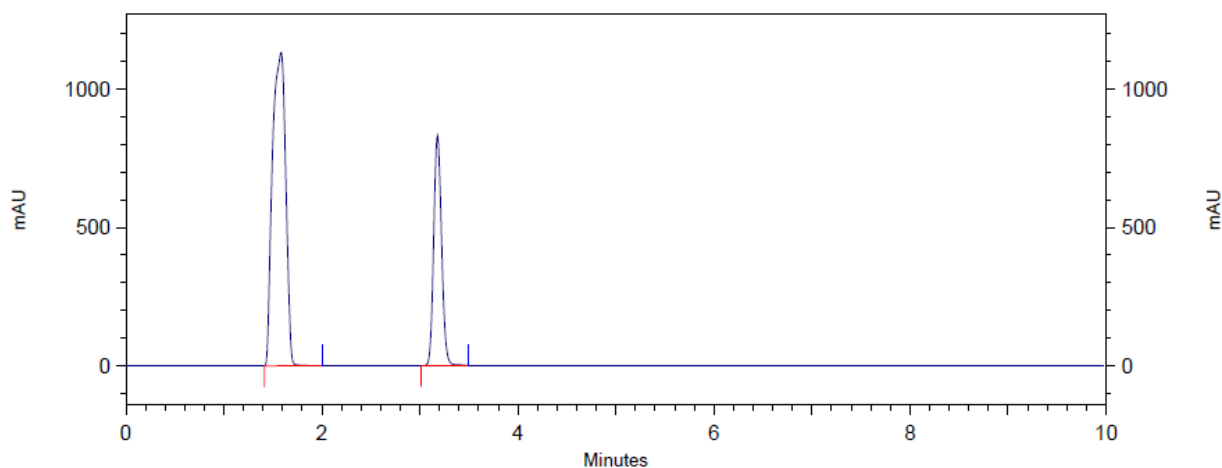


Figure 2 Final Chromatogram of Remogliflozin etabonate and Vildagliptin (M.P:-Buffer:Methanol:ACN (35:55:10)%v/v with pH 3.5)

7.2 FORCED DEGRADATION:-Sample were injected under various stress conditions. Here , chromatograms of optimized degradation conditions are shown

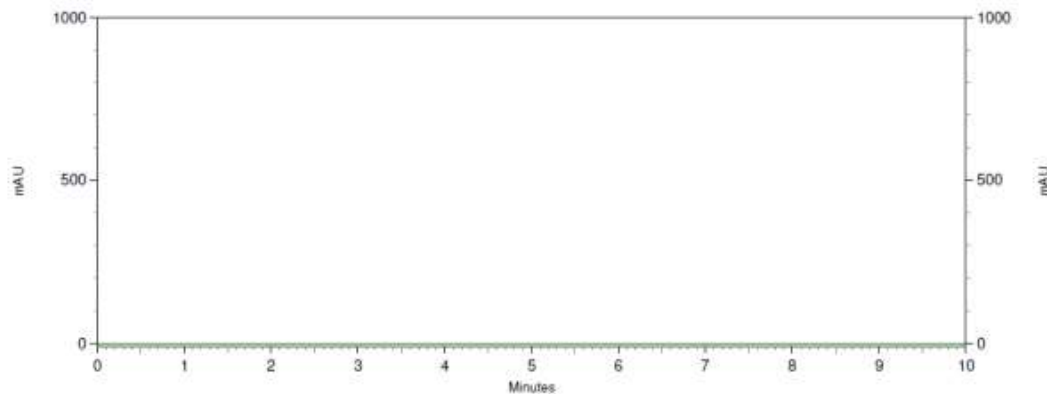


Figure 3 Chromatogram of blank solution

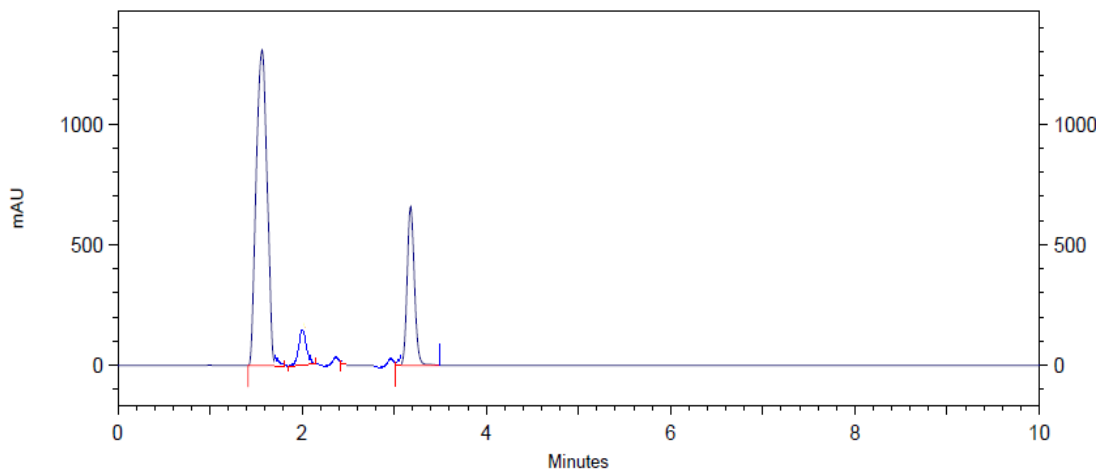


Figure 4 Chromatogram of Sample (300ppm Remo and 150ppm Vilda) for Acid Degradation

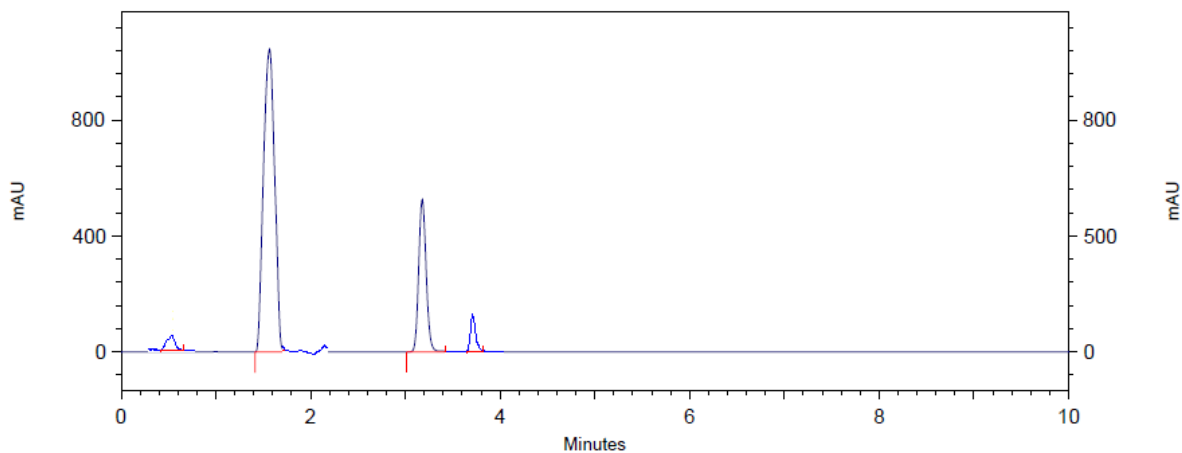


Figure 5 Chromatogram of Sample (300ppm Remo and 150ppm Vilda) for Base Degradation

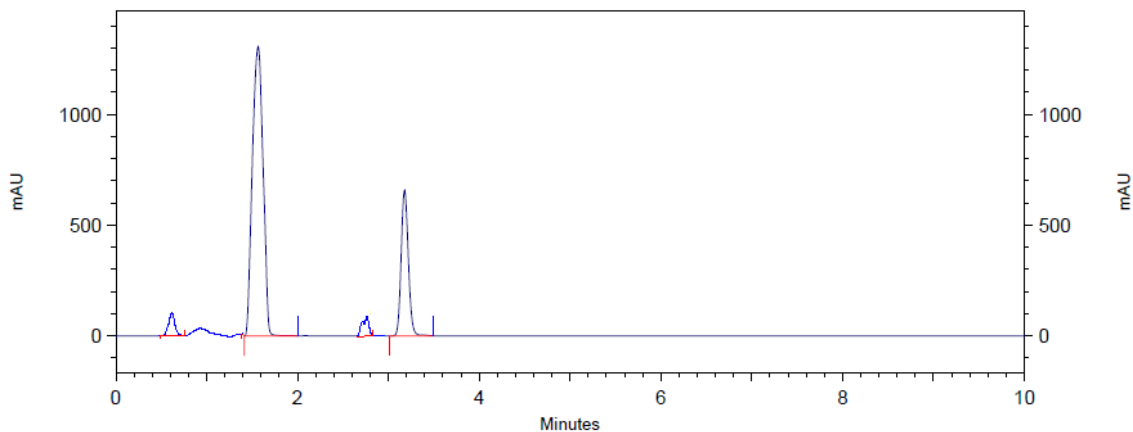


Figure 6 Chromatogram of Sample (300ppm Remo and 150ppm Vilda) for H₂O₂ Degradation

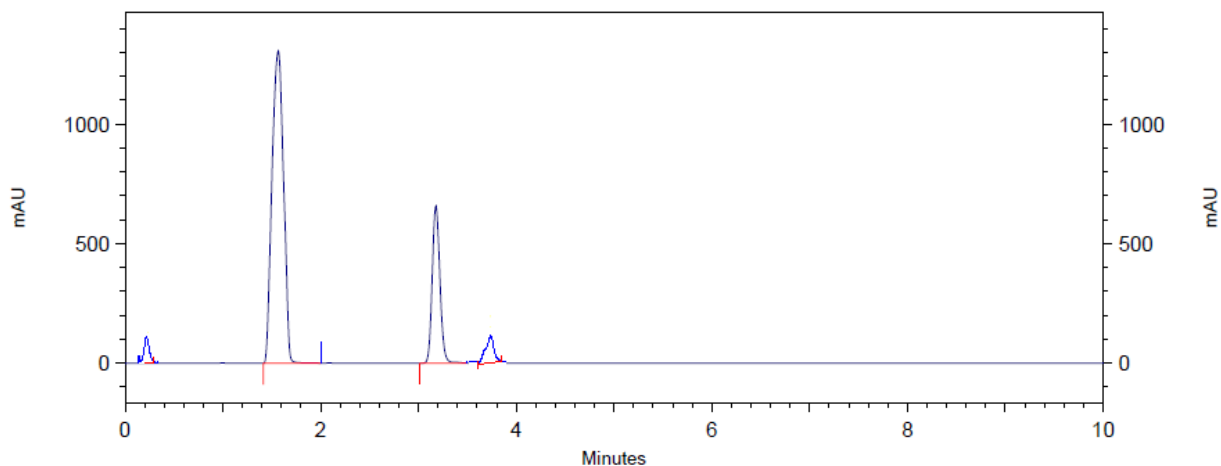


Figure 7 Chromatogram of Sample (300ppm Remo and 150ppm Vilda) for Thermal Degradation

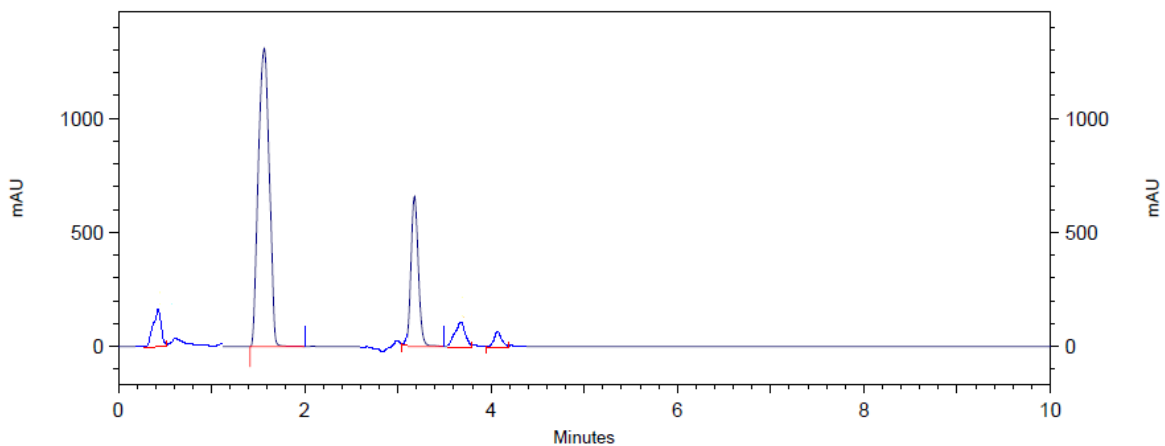


Figure 8 Chromatogram of Sample (300ppm Remo and 150ppm Vilda) for Photo Degradation

Type of Degradation	Solutions		Area		%Degradation	
As such	Remogliflozin		21714220		-	
	Vildagliptin		7410385		-	
	Remogliflozin	Vildagliptin	22479745	7573248	-	-
Acid 1N Hcl for 1hr at room temperature	Remogliflozin		20333025		6.37	
	Vildagliptin		7025102		5.2	
	Remogliflozin	Vildagliptin	21098550	7119743	6.15	5.99
Base 1N NaOH for 1hr at room temperature	Remogliflozin		18990157		12.55	
	Vildagliptin		6490803		12.41	
	Remogliflozin	Vildagliptin	19720314	6597520	12.28	12.89
H ₂ O ₂ 3% H ₂ O ₂ for 1hr at room temperature	Remogliflozin		20980750		3.38	
	Vildagliptin		7258811		2.05	
	Remogliflozin	Vildagliptin	21746275	7421674	3.27	2.01
Thermal At 60 ⁰ C for 2hrs	Remogliflozin		21386493		1.51	
	Vildagliptin		7382619		0.38	
	Remogliflozin	Vildagliptin	22152018	7520921	1.46	0.7
Photo In Sun light for 1hr	Remogliflozin		20596846		5.15	
	Vildagliptin		7296059		1.55	
	Remogliflozin	Vildagliptin	21362371	7449968	4.98	1.63

8. Validation

Various validation parameters were applied for developed method. Chromatograms of validation are shown here.

8.1 System Suitability Test

Table 3 System Suitability Data for Remogliflozin etabonate(300µg/ml)

Sr.No.	Retention Time	Area	Tailing Factor	Theoretical Plate
1	1.567	22481678	0.978	7040
2	1.568	22498657	0.975	7063
3	1.567	22494024	0.974	7103
4	1.567	22497897	0.975	7078
5	1.566	22489597	0.970	7021
6	1.567	22489550	0.973	7053

Table 4 System Suitability Data for Vildagliptin(150µg/ml)

Sr.No.	Retention Time	Area	Tailing Factor	Theoretical Plate
1	3.180	7599023	1.109	7805
2	3.180	7598832	1.102	7823
3	3.181	7591503	1.102	7897
4	3.180	7598128	1.108	7800
5	3.181	7608856	1.103	7842
6	3.180	7608670	1.109	7853

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Table 5 System Suitability Parameter

Parameter	Observation		Specification
	Remogliflozin etabonate	Vildagliptin	
%RSD of Area	0.03	0.09	RSD<2%
Tailing factor(T)	0.974	1.105	T≤2
Theoretical plates(N)	7059	7836	≥2000

Observation: The system suitability parameters were well within acceptance criteria, which indicate that the system and chromatographic conditions are suitable for this method.

8.2 Specificity

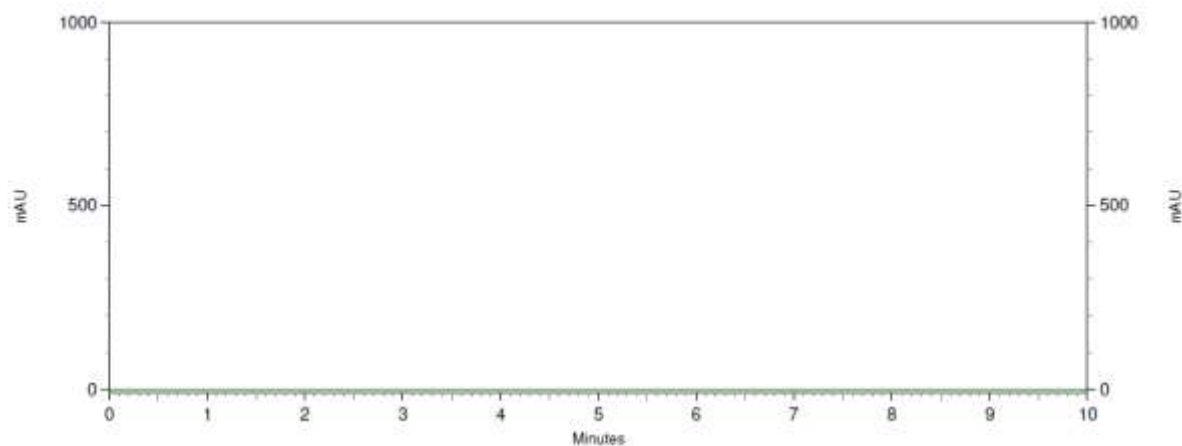


Figure 9 Chromatogram of Blank

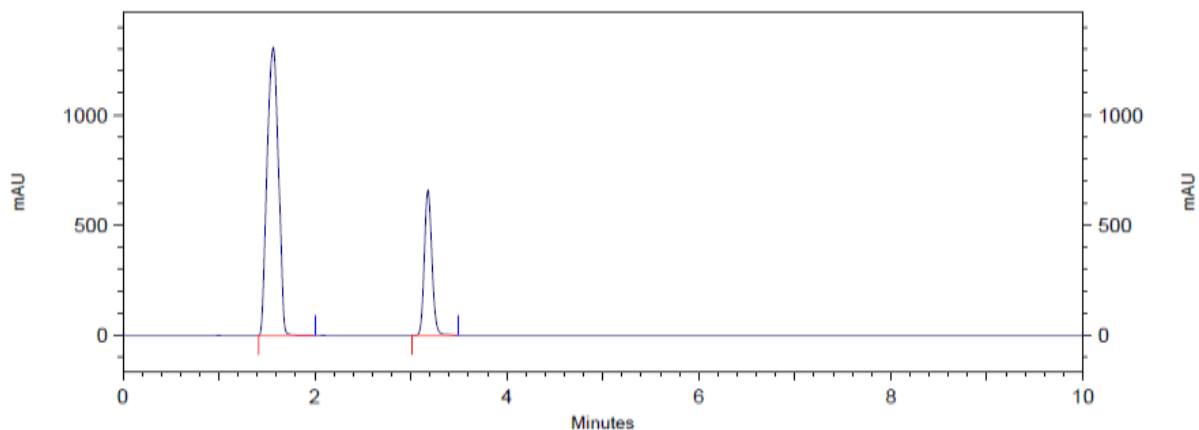


Figure 10 Chromatogram of Sample (300 μ g/ml Remogliflozin etabonate and 150 μ g/ml Vildagliptin)

Observation:-

The Chromatograms of Remogliflozin etabonate and Vildagliptin sample show no interference with the chromatogram of Remogliflozin and Vildagliptin blank, so the developed method is specific.

8.3 Linearity and Range

Table 6 Data of peak areas of Remogliflozin etabonate (150-450 μ g/ml)

Sr. No.	Concentration (μ g/ml)	Peak area of Sofosbuvir
1	150	11985674
2	240	17881640
3	300	22478245
4	360	26973894
5	450	32765039

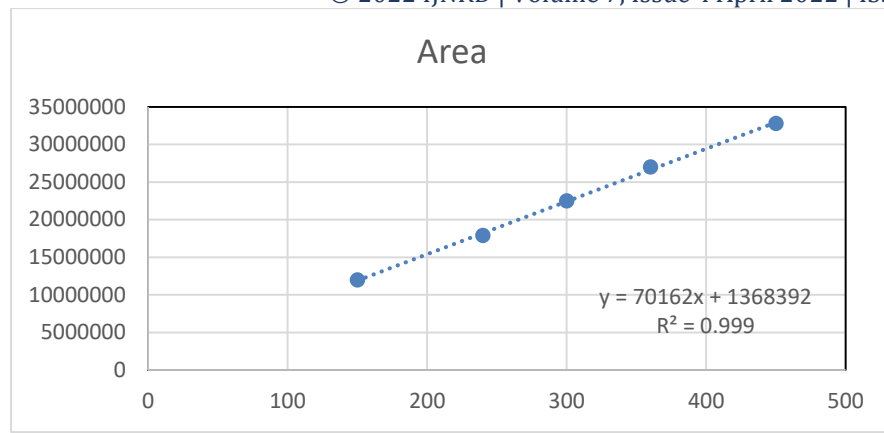


Figure 11 Linearity Curve of Remogliflozin etabonate

Table 7 Data showing regression characteristics of Remogliflozin etabonate

Regression equation	$Y = 70162x + 1368392$
Regression co-efficient	0.999

Table 8 Data of peak areas of Vildagliptin (75-225µg/ml)

Sr. No.	Concentration (µg/ml)	Peak area of Sofosbuvir
1	75	3925985
2	120	6136489
3	150	7572764
4	180	8989305
5	225	11355600

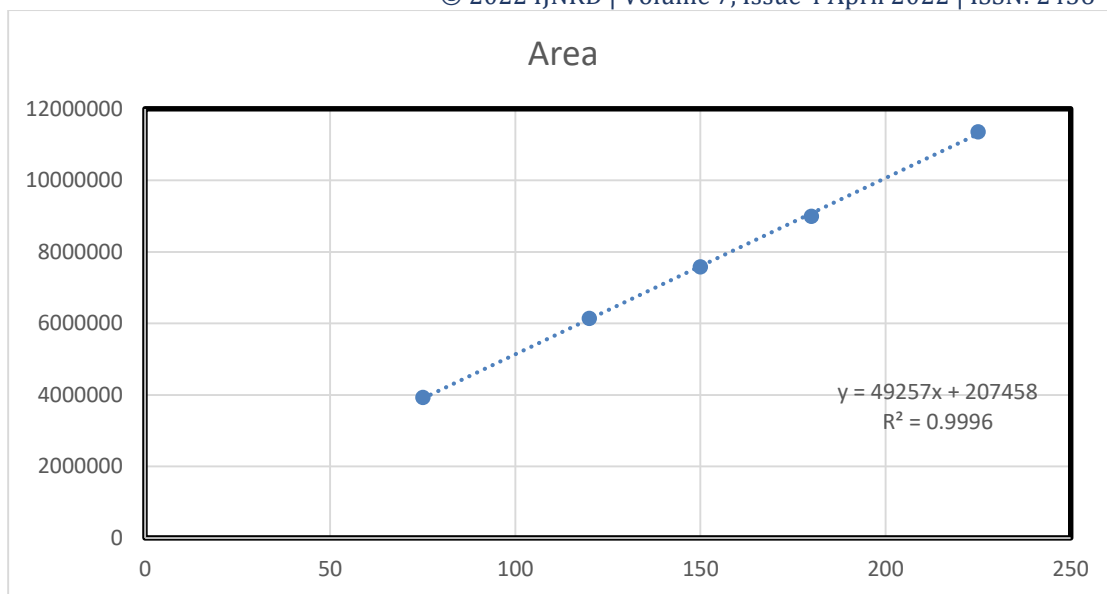


Figure 12 Linearity Curve of Vildagliptin

Table 9 Data showing regression characteristics of Vildagliptin

Regression equation	$Y = 49257x + 20745$
Regression co-efficient	0.999

Observation:- The area obtained were directly proportional to the concentration of analyte in the sample. The method can, therefore be termed as linear in specific range.

8.4 Precision

Intraday Precision: Intraday Precision was performed by analyzing three different concentrations within linearity range, three times in a day (3*3 determination).

Interday Precision: Interday Precision was performed by analyzing three different concentration within linearity range, on different days.

Repeatability: The repeatability studies were carried out by measuring response for a single concentration for six times a day.

Table 10 Intraday Precision Data for Remogliflozin etabonate and Vildagliptin

Remogliflozin etabonate			Vildagliptin		
Conc (µg/ml)	Area±S.D.(n=3)	%RSD	Conc (µg/ml)	Area±S.D.(n=3)	%RSD
150	11742877±63141.2066	0.54	75	3915023±22085.7810	0.56
300	22487940±9408.0967	0.04	150	7505481±5591.4989	0.07
450	33122164±589345.1636	1.78	225	11258934±23685.6268	0.21

Table 11 Interday Precision Data for Remogliflozin etabonate and Vildagliptin

Remogliflozin etabonate			Vildagliptin		
Conc (µg/ml)	Area±S.D.(n=3)	%RSD	Conc (µg/ml)	Area±S.D.(n=3)	%RSD
150	11807383±59984.8455	0.51	75	39900201±14604.6993	0.37
300	22490072±8757.2087	0.04	150	7599360±516.3787	0.01
450	32916417±117523.65	0.36	225	11285076±14738.97	0.13

Table 12 Repeatability Data for Remogliflozin etabonate and Vildagliptin

Sr No.	Remogliflozin etabonate (300µg/ml)Area±SD	Vildagliptin (150µg/ml)Area±SD
1	22481278	7599568
2	22498457	7598569
3	22490254	7599475
4	22497489	7598547
5	22489597	7608548
6	22489819	7608230

Mean	22491148	7608548
S.D.	6260.2613	4848.2594
%RSD	0.03	0.06

Observation:-

- **Intraday Precision:-** The %RSD was found to be 0.54-1.78% for Remogliflozin etabonate and 0.56-0.21% for Vildagliptin. These %RSD value was found to be less than ± 2.0 indicated that the method is precise.
- **Interday Precision:-** The %RSD was found to be 0.51-0.36% for Remogliflozin etabonate and 0.37-0.13% for Vildagliptin. These %RSD value was found to be less than ± 2.0 indicated that the method is precise.
- **Repeatability:-** The % RSD was found to be 0.03% for Remogliflozin etabonate and 0.06% for Vildagliptin. . These %RSD value was found to be less than ± 2.0 indicated that the method is precise.

8.5 Accuracy

Recovery study include addition of standard drug to the sample at 3 different concentration levels (50%,100%,150%) taking into % purity of added bulk drug sample. At each concentration,sample was injected thrice to check repeatability and from the % RSD values it was analyzed that the method was accurate as % recovery values found to be in the range of 100% for Remogliflozin etabonate and 99.94-100.20% for Validation at different concentrations 50%,100%.150%.

Table 13 Recovery Data for Remogliflozin etabonate

Conc. Level (%)	Sample amount ($\mu\text{g/ml}$)	Amount Added($\mu\text{g/ml}$)	Amount Recovered ($\mu\text{g/ml}$)	%Recovery	%Mean Recovery \pm SD
50%	300	150	148.61	99.078	100 \pm 0.8587
	300	150	148.72	99.150	
	300	150	150.90	100.60	
100%	300	300	301.16	100.38	100 \pm 0.5756
	300	300	297.71	99.239	
	300	300	299.25	99.751	
150%	300	450	447.48	99.442	100 \pm 0.4229
	300	450	450.97	100.21	

	300	450	450.55	100.12	
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Table 14 Recovery Data for Vildagliptin

Conc. Level (%)	Sample amount (µg/ml)	Amount Added(µg/ml)	Amount Recovered (µg/ml)	%Recovery	%Mean Recovery±SD
50%	150	75	74.964	99.952	100.20±0.4333
	150	75	74.967	99.957	
	150	75	75.529	100.705	
100%	150	150	150.075	100.050	100.04±0.1411
	150	150	149.850	99.900	
	150	150	150.273	100.182	
150%	150	225	225.107	100.047	99.94±0.2152
	150	225	225.200	100.089	
	150	225	224.318	99.697	

Observation:- The result of this study was found to be within the acceptance criteria of method validation (i.e. the recovery is 99.078-100.182 % and the RSD is NMT 2.0%), this proves that the test method is accurate for the estimation of Remogliflozin etabonate and Vildagliptin Tablet.

8.6 Robustness

Table 15: Robustness data for ± 0.2 mL/min variation in Flow rate

Flow rate	Peak area		Mean \pm SD		% RSD	
	Remogliflozin etabonate	Vildagliptin	Remogliflozin etabonate	Vildagliptin	Remogliflozin etabonate	Vildagliptin
0.8 mL/min	22497820	7603448	22437175 \pm	7601082 \pm	0.38	0.04
	22376529	7598715	85765.68	3346.73		
1.2 mL/min	22498480	7599478	22477788 \pm	7595150 \pm	0.13	0.08
	22457095	7590821	29263.61	6121.42		

Table 16: Robustness data for $\pm 2\%$ variation in Temperature

Temperature (°C)	Peak area		Mean \pm SD		% RSD	
	Remogliflozin etabonate	Vildagliptin	Remogliflozin etabonate	Vildagliptin	Remogliflozin etabonate	Vildagliptin
28°C	22494156	7602445	22493993 \pm 23	7601866 \pm 81	0.00	0.01
	22493829	7601286	1.22	9.53		
32°C	22494597	7603405	22489361 \pm 74	7603762 \pm 50	0.03	0.01
	22484125	7604119	04.82	4.87		

Observation:-

Theoretical plates and Asymmetry values are from the first injection of the system suitability set were found well within the acceptance criteria as per system suitability. So, the study proves the reliability of test method for minor changes in chromatographic condition. Hence method can be termed as robust.

8.7 LOD and LOQ

Calculation curve was repeated for five times and standard deviation (SD) of the intercepts was calculated. Then LOD and LOQ were calculated as follows:

- $LOD = 3.3 \times SD/slope$
- $LOQ = 10 \times SD/slope$

Where, SD= Standard deviation of intercepts

Limit of Detection**Table 17 limit of detection data for Remogliflozin etabonate and Vildagliptin**

Remogliflozin etabonate	Vildagliptin
$LOD = 3.3 \times (SD/Slope)$ $LOD = 3.3 \times (114.23/70162)$ $LOD = 0.0053 \mu\text{g/ml}$	$LOD = 3.3 \times (SD/Slope)$ $LOD = 3.3 \times (57.11/49257)$ $LOD = 0.0038 \mu\text{g/ml}$

Limit of Quantification**Table 18 limit of detection data for Remogliflozin etabonate and Vildagliptin**

Remogliflozin etabonate	Vildagliptin
$LOD = 10 \times (SD/Slope)$ $LOD = 10 \times (114.23/70162)$ $LOD = 0.016 \mu\text{g/ml}$	$LOD = 10 \times (SD/Slope)$ $LOD = 10 \times (57.11/49257)$ $LOD = 0.0011 \mu\text{g/ml}$

8.8 Applicability of Method for Marketed Formulation

Analysis of the pharmaceutical formulation (tablet) was done by the proposed method and the % Assay was calculated. Sample solution was prepared as per above. The quantification was carried out by keeping these values to the straight line equation of calibration curve.

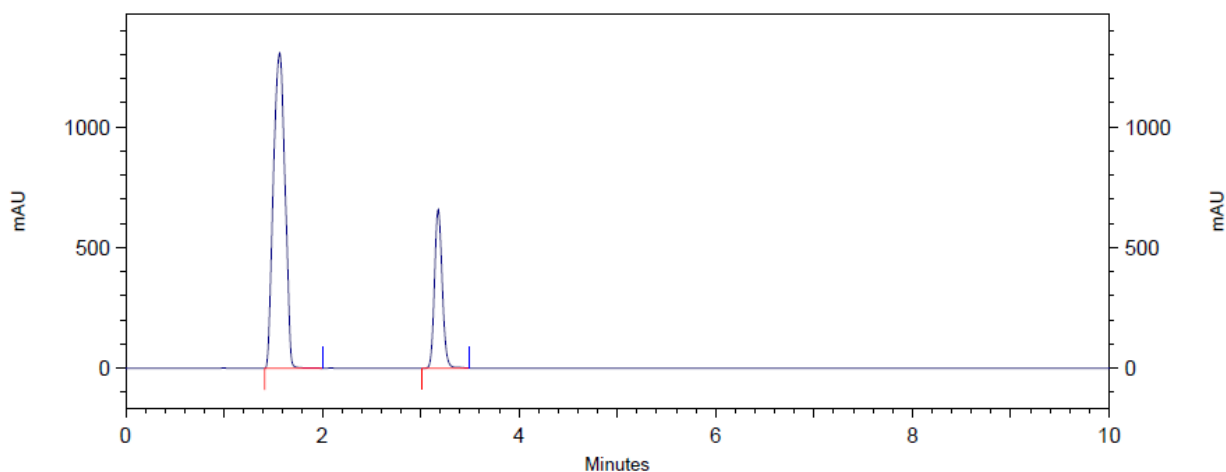


Figure 13 Chromatogram of Tablet Sample Solution

Table 19 Analysis of Marketed Formulation of Remogliflozin etabonate and Vildagliptin by proposed method

Sample No.	Lable Claim ($\mu\text{g/ml}$)		Amount Found ($\mu\text{g/ml}$)		%Assay	
	Remogliflozin etabonate	Vildagliptin	Remogliflozin etabonate	Vildagliptin	Remogliflozin etabonate	Vildagliptin
1	100	50	99.45	49.50	99.45	99
2	100	50	99.34	49.61	99.34	99.22
3	100	50	99.36	49.50	99.36	99
Mean			99	50	99	99
Standard Deviation			0.0586	0.0635	0.058	0.127
%RSD					0.06	0.13

Observation:-

By RP-HPLC method %assay was found 99 and 99 for Remogliflozin etabonate and Vildagliptin respectively. So the developed method can be used for routine analysis.

6.6 Method Validation Summary

Parameters		Remogliflozin etabonate	Vildagliptin
Specificity		Specific	
Linearity and range		150-450µg/ml	75-225µg/ml
Precision (%RSD)	Repeatability	0.03	0.06
	Intraday	0.54-1.78	0.56-0.21
	Interday	0.51-0.36	0.37-0.13
Accuracy	50%	100±0.858	100.20±0.433
	100%	100±0.5756	100.04±0.1411
	150%	100±0.4229	99.94±0.2152
Robustness		The system suitability parameters were found well within the acceptance criteria as per system suitability.	
Limit of Detection		0.0053µg/ml	0.0038µg/ml
Limit of Quantitation		0.016µg/ml	0.0011µg/ml
% Assay		0.06	0.13

8.9 DISCUSSION

A new stability indicating RP-HPLC method has been developed for simultaneous estimation of Remogliflozin etabonate and Vildagliptin in tablet dosage form was rapid, accurate, precise, specific, sensitive, economic and easy to perform. From the above study we can conclude that the Remogliflozin etabonate and Vildagliptin undergo degradation to different extent under different stress conditions. In this study, the % degradation for each type of degradation was competent. From the specificity studies, it was confirmed that the peak of the degradation product and excipient was not interfering with the peak of drugs. Hence, this method can be used for analysis of Remogliflozin etabonate and Vildagliptin in bulk drug and pharmaceutical dosage form in quality control department for routine analysis. Linearity of the developed method was near to 1, range was found 150-450µg/ml for Remogliflozin etabonate and 75-225µg/ml for Vildagliptin. %RSD was found to be less than 2 for precision. %Recoveries were found in range of 99.07-100.38% and 99.69-100.70% for Remogliflozin etabonate and

Vildagliptin respectively. Hence, this method can be used for analysis for Remogliflozin etabonate and Vildagliptin in bulk drug and pharmaceutical dosage form in quality control department for routine analysis.

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