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DEVELOPMENT AND VALIDATION OF UV SPECTROPHOTOMETRIC METHOD AND RP – HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF DAPAGLIFLOZIN PROPANEDIOL AND GLIMEPIRIDE IN SYNTHETIC MIXTURE

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ABSTRACT

An accurate, precise and reproducible UV-spectrophotometric methods and liquid chromatographic assay method were developed and validated for the determination of Dapagliflozin propanediol and Glimepiride in synthetic mixture. Spectrophotometric estimation was done by derivative spectroscopic method and methanol as solvent. In this method λmax for Dapagliflozin propanediol and Glimepiride were selected at 288 nm and 224nm. RP-HPLC analysis was carried out using Pearless C-18 column (4.6 x 250mm, 5µ particle size) and mobile phase composed of Acetonitrile : 10% Ortho-phosphoric acid in water pH 6.0 (70:30% v/v)at a flow rate of 1.0 ml/min and chromatogram was recorded at 228 nm. Linearity was evaluated over the concentration range of 5 -30 µg/ml and 5-30 µg/ml for Dapagliflozin propanediol and Glimepiride in UV spectrophotometric and in RP-HPLC method Linearity was evaluated over the concentration range of 1 -5 µg/ml and 1-5 µg/ml for Dapagliflozin propanediol and Glimepiride (the value of r2 = 0.9978 and r2= 0.995 found were by UV method for DAPA and GLM and the value of r2 = 0.997 and r2= 0.996 found were by RP-HPLC method for DAPA and GLM). The developed methods were validated according to ICH guidelines and values of accuracy, precision and other statistical analysis were found to be in good accordance with the prescribed values therefore the both methods can be used for routine monitoring of DAPA and GLM in the assay of Synthetic mixture of both drugs.

KEYWORDS: Dapagliflozin propanediol and Glimepiride in synthetic mixture.

INTRODUCTION

Dapagliflozin is indicated for the management of diabetes mellitus type2, and function to improve glycemic control in adults when combined with diet and exercise. Dapagliflozin is a sodium-glucose co-tranporter – inhibitor, which prevent glucose reabsorption in the kidney. Using dapagliflozin leads to heavy glycosuria, which can lead to weight loss and tiredness. Dapagliflozin is not recommended for patients with type 1 diabetes mellitus or for the treatment of diabetic ketoacidosis.

Glimepiride is used to treat high blood sugar levels caused by type 2 diabetes. It may be used alone, or in combination with insulin or another oral medicine such as metformin. In type 2 diabetes, insulin produced by the pancreas is not able to get sugar into the cells of the body where it can work properly. Using this medicine will help lower blood sugar when it is too high and help restore the way you use food to make energy. . Some people can control type2 diabetes with diet alone or diet and exercise. Following a specially planned diet and exercising will always be important when you have diabetes, even when you are taking medicines. To work properly, the amount of glimepiride you take must be balanced against the amount and type of food you eat and the amount of exercise you do. If you change your diet, your exercise, or both, you will want to test your blood sugar to find out if it is too low.

From the literature survey it was found that few analytical method like UV Spectrophotometric, high performance liquid (HPLC), Reverse phase high performance liquid chromatography (RP-HPLC), LC-MS and chromatography methods have been reported for simultaneous determination of Dapagliflozin and Glimepiride in combination.

There is no any single UV method and RP-HPLC method reported for simultaneous analysis of Dapagliflozin and Glimepiride. A successful attempt has been made to estimate two drugs simultaneously by first derivative spectroscopy and RP-HPLC. The objective of the investigation is to develop and validate an analytical method for the estimation of Dapagliflozin Propanediol and Glimepiride in a combined mixture by first derivative spectroscopic method and RP-HPLC.

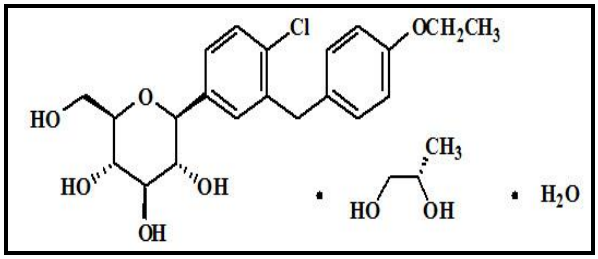

Figure 1: Dapagliflozin Propanediol structure.

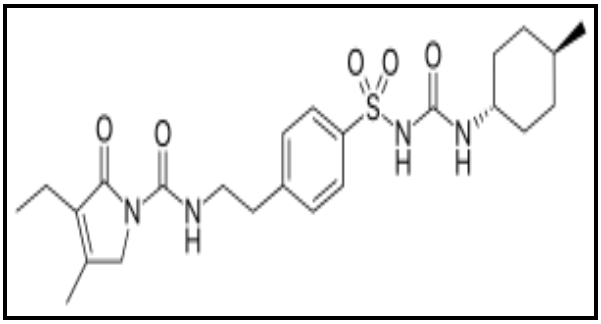

Figure 2: Glimepiride Structure.

- **U.V Spectrophotometric Methods**
- **Identification of Pure Drugs (A.P.I)**
- **Experimental**
- **Instrumentation**
- Melting Point Apparatus
- FT-IR : Model- Miracle -10, single reflection ATR accessory, Shimadzu
- Digital Analytical Balance- Wensar DA13-220
- U.V. Visible Spectrophotometer: A Shimadzu model 1800 (Japan) With software UV Probe (version 2.31)

- **Reagents and materials**
- Dapagliflozin Propanediol and Glimepiride were supplied by shree Parikh trading, Ahmedabad and West-Coast Pharma, Ahmedabad, India Respectively.
- Methanol AR grade.

- **Identification by Melting point determination**
- Melting point of Dapagliflozin Propanediol and Glimepiride has been determined by using Melting Point Apparatus. The melting points of the compounds were taken by open capillary method. It is shown in Table 4.1.

Table 1: Melting Point of Drugs.

Sr. no	Drug	Reported melting point[4,5]	Observed melting point
1	Dapagliflozin propanediol	65-70 ^{O}C	67-68 ^{O}C
2	Glimepiride	206-208^{O}C	207-208 ^{O}C

- **Identification by I.R Spectroscopy**
- **Identification of Dapagliflozin Propanediol by I.R. Spectroscopy**

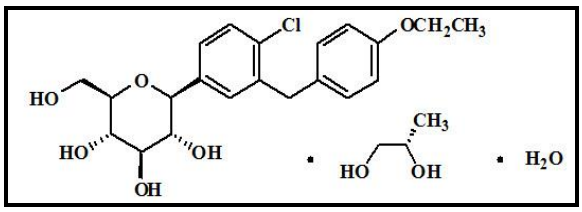

Figure 3: Dapagliflozin Propanediol structure.

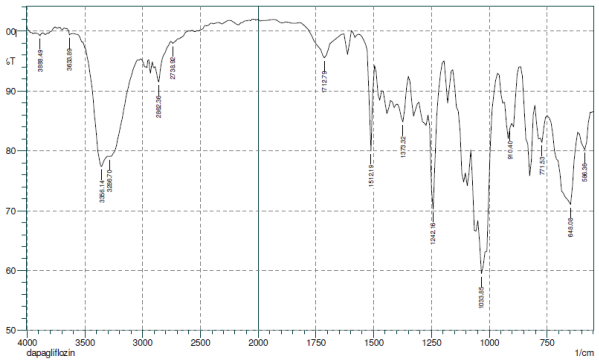

Figure 4: Dapagliflozin Propanediol Sample I.R. spectra.

Table 2: Interpretation of FT-IR spectra of Dapagliflozin propanediol.[55]

Sr. No.	Functional group Characteristic	Standard Absorption (cm^{-1})	Observed Absorption (cm^{-1})
1	C-Cl (s)	800-600	648.08
2	C-O (s)	1050-1150	1033.65
3	C=C (s)	1400-1600	1512.19
4	C-H (s)	2900-2820	2862.36
5	O-H (s)	3400-3200	3356.14

- **Identification of Glimepiride by I.R Spectroscopy**

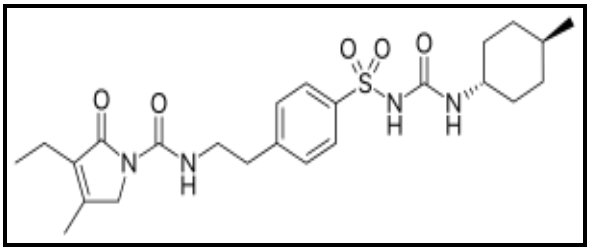

Figure 4.3: Glimepiride Structure.

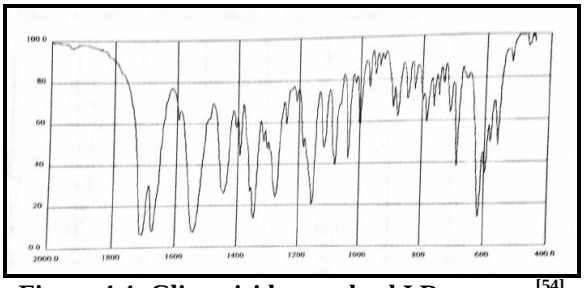

Figure 4.4: Glimepiride standard I.R. spectra[54]

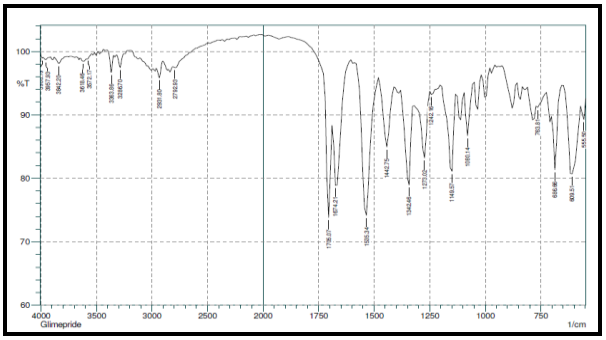

Figure 5: Glimepiride sample I.R. Spectra.

Table 3: Interpretations of FT-IR Spectra of Glimepiride[55]

Sr. No.	Functional Group	Standard frequency (cm^{-1})	Observed frequency (cm^{-1})
1	S=O (s)	1140-1160	1149.57
2	C-N (s)	1080-1360	1342.46
3	C=C (s)	1670-1675	1674.21
4	C=O (s)	1725-1705	1705.07
5	N-H (s)	3300-3500	3363.66

❖ **Identification By UV Spectroscopy Method**

Table 4: λmax of Dapagliflozin propanediol and Glimepiride.

Drug name	Observed λmax (Methanol)	Reported λmax (Methanol)[28]
Dapagliflozin propanediol	224 nm	223 nm
Glimepiride	228 nm	228 nm

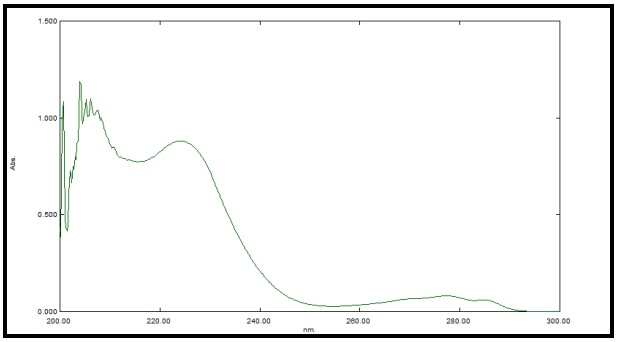

Fig 6: Dapagliflozin propanediol UV Spectra at 224 nm (20 µg/ml).

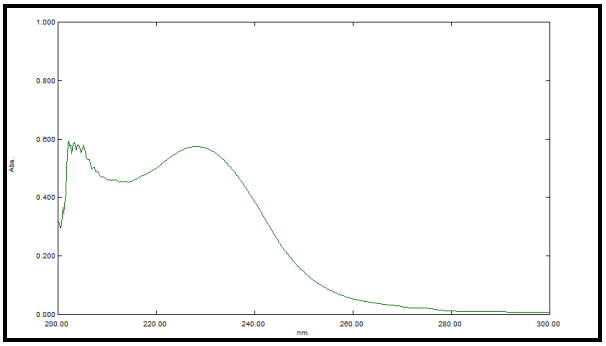

Fig 7: Glimepiride UV Spectra at 228 nm (20 µg/ml).

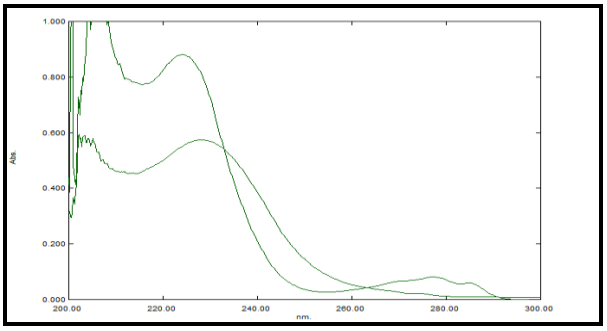

Fig 8: Overlain Spectra of DAPA at 224 nm (20 µg/ml) and GLM.

At 228 nm (20 µg/ml) in Methanol

➢ **Solubility study**

The solubility study of Dapagliflozin propanediol and Glimepiride were determined by taking 10 mg of both drug in 10 ml volumetric flasks, shaken for few minutes and add the required quantity of solvent for complete solubility.

Table 5: Solubility of Dapagliflozin propanediol and Glimepiride.

Solvent	Dapagliflozin propanediol	Glimepiride
Distilled Water	Soluble	Insoluble
Methanol	Soluble	Soluble

❖ **U.V. Spectrophotometric Method**

❖ **First-order derivative method for Dapagliflozin propanediol and Glimepiride**

- **Experimental work**

➢ **Instrument and Apparatus**

- UV Visible Spectrophotometer: Shimadzu model 1800
- Digital Analytical balance – Wensar DAB – 220
- Sonicator- Equitron
- Volumetric Flask- 10,50,100 ml (Borosilicate)
- Measuring Cylinder- 10,50,100 ml (Borosilicate)

➢ **Chemical and Reagents**

- Dapagliflozin propanediol
- Glimepiride
- Methanol (AR grade) was used as a solvent throughout the experimentation.

➢ **Solvent selection**

- **Solvent :** Methanol

❖ **Spectrophotometric conditions**

- **Mode:** Absorption (scanning)
- **Scan Speed**: Medium
- **Wavelength Range:** 200-400nm
- **Initial Baseline Correction:** Methanol(AR grade)

❖ **Preparation of solutions**

❖ **Preparation of standard stock solutions**

- **Preparation of standard stock solution of Dapagliflozin propanediol (1000µg/ml):**

Accurately weight 100 mg of Dapagliflozin propanediol was transferred into a 100 ml volumetric flask and diluted with Methanol.

- **Preparation of standard stock solution of Glimepiride (1000µg/ml):**

Accurately weight 100 mg of Glimepiride was transferred into a 100 ml volumetric flask and diluted with Methanol.

❖ **Preparation of working standard solutions**

- **Preparation of working standard stock solution of Dapagliflozin propanediol (100µg/ml)**

10 ml standard stock solution of Dapagliflozin propanediol was transferred in 100 ml volumetric flask and dilute up to mark with methanol.

- **Preparation of working standard stock solution of Glimepiride (100µg/ml)**

10 ml standard stock solution of Glimepiride was transferred in 100 ml volumetric flask and dilute up to mark with methanol.

❖ **Selection of wavelength for measurement**

➢ 3 ml working standard stock solution of Dapagliflozin propanediol (100µg/ml) and 3 ml working standard stock solution of Glimepiride (100µg/ml) was transferred in separate 10 ml volumetric flask and dilute up to mark with methanol to get the 30 µg/ml of Dapagliflozin propanediol and 30 µg/ml of Glimepiride. Each solution scanned in the range of 200-400 nm.

❖ **Preparation of calibration curve**

➢ **Calibration curve for Dapagliflozin propanediol**

❖ Aliquots of stock solution of Dapagliflozin propanediol (100 µg/ml) 0.5, 1, 1.5, 2, 2.5, 3 were pipette out in 10 ml volumetric flask separately and dilute up to the mark with Methanol which will give 5-30 µg/ml solution was prepared and absorbance was measured at 288 nm in U.V.

❖ Absorbance of each solution was measured at 288 nm using methanol as blank. Calibration was obtained by plotting respective absorbance against concentration in µg/ml and the regression equation was computed.

➢ **Calibration curve for Glimepiride**

❖ Aliquots of stock solution of Glimepiride (100 µg/ml) 0.5, 1, 1.5, 2, 2.5, 3 ml were pipette out in 10 ml volumetric flask separately and dilute up to the mark with Methanol which will give 5-30 µg/ml solution was prepared and absorbance was measured at 224 nm in U.V. Absorbance of each solution was measured at 224 nm using methanol as blank. Calibration was obtained by plotting respective absorbance against concentration in µg/ml and the regression equation was computed.

❖ **Assay**

❖ **Preparation of Synthetic Mixture of Dapagliflozin propanediol and Glimepiride**

- The Synthetic Mixture of Dapagliflozin propanediol and Glimepiride was prepared in ratio of 1:1.
- excipients, Microcrystalline Cellulose, starch, Magnesium Stearate, lactose, colloidal silicon dioxide along with the drug Dapagliflozin propanediol 10 mg and Glimepiride 10 mg.
- Accurately weighed equivalently weight of Dapagliflozin propanediol (10 mg) and Glimepiride (10 mg) which transferred in 100 ml volumetric flask and make up half mark with Methanol. This solution was Sonicated till the drug dissolves and was made up to mark with Methanol. Then this solution was filtered through Whatmann filter paper. So, obtained concentration of Dapagliflozin propanediol is 100 µg/ml and Glimepiride is 100 µg/ml.

❖ **Preparation of Working Sample Solution**

Accurately 1 ml of the above solutions was pipette out into 10 ml volumetric flask and the volume was adjusted up to the mark with Methanol to make final concentration Dapagliflozin propanediol was 10 µg/ml and Glimepiride 10 µg/ml. Absorbance of resulting solution was measured at 288 nm and 224 nm.

❖ **Validation**

➢ The developed method was validated with respect to linearity, accuracy, intraday and interday precision, limit of detection (LOD) and limit of quantification (LOQ) and robustness in accordance with the ICH guideline.

❖ **Linearity and Range**

➢ Linearity was studied by preparing standard solution at 6 different concentrations. The linearity range for Dapagliflozin propanediol and Glimepiride were found to be 5-30 µg/ml and 5-30 µg/ml respectively. Linearity was assessed in the term of slope, intercept and correlation coefficient for both drugs.

❖ **Precision**

➢ The precision of analytical procedure express 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 may be considered at three levels: repeatability, intermediate (intraday precision) and reproducibility (interday precision)

1) **Intraday precision:** Solution containing 5, 10, 15 µg/ml of DAPA and 5, 10, 15 µg/ml GLM were analysed three times on the same day and %R.S.D was calculated.
2) **Interday precision:** Solution containing 5, 10, 15 µg/ml of DAPA and 5, 10, 15 µg/ml GLM were

analysed three different successive days and %R.S.D was calculated.

3) **Repeatability:** Method precision of experiment was performed by preparing the standard solution of DAPA (10 µg/ml) and GLM (10 µg/ml) for six times and analysed as per the proposed method. % R.S.D was not more than 2 %.

❖ **Limit of Detection (LOD)**

➢ Limit of Detection can be calculated using following equation as per ICH guidelines.

$$LOD = 3.3 \times (N / S)$$

Where, σ = the standard deviation of the response
S= the slope of the calibration curve.

❖ **Limit of Quantification (LOQ)**

Limit of Quantification can be calculated using following equation as per ICH guidelines.

$$LOD = 10 \times (N / S)$$

Where, σ = the standard deviation of the response
S= the slope of the calibration curve.

❖ **Accuracy**

➢ Accuracy expresses the closeness of agreement between the value which is accepted either as a conventional true value or accepted reference value and the value found. Recovery studies were carried out by addition of standard drug to the sample at 3 different concentration levels (50%, 100%, 150%) taking into consideration percentage recovery of added bulk drug sample. The experiment was repeated three times by spiking previously analysed sample of tablet with three different concentrations of standards.

❖ **RESULT AND DISCUSSION**

❖ **Selection of wavelength for simultaneous estimation of Dapagliflozin propanediol and Glimepiride**

➢ The zero-order crossing point of Dapagliflozin propanediol (30µg/ml) and Glimepiride (30 µg/ml).it is evident that Dapagliflozin propanediol and Glimepiride shows ZCP point at 288 nm And 224 nm.

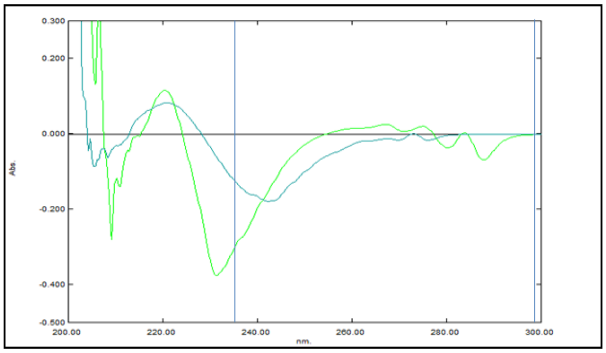

Fig. 9: Zero crossing point of Dapagliflozin propanediol at 288 nm (30µg/ml) and Glimepiride at 224 nm (30µg/ml).

❖ **Method validation**

➢ **Linearity and Range**

The linearity of DAPA and GLM was found to be in the range of 5-30 µg/ml and 5-30 µg/ml respectively.

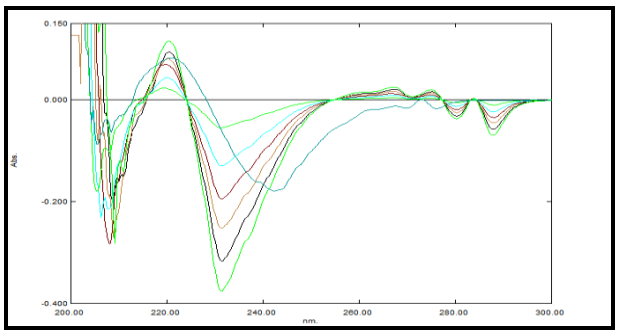

Fig 10: First order Spectra of Dapagliflozin propanediol in Methanol at 288 nm (5-30 µg/ml).

Table 6: Linearity data for Dapagliflozin propanediol.

Sr No	Concentration (µg/ml)	Mean Absorbance ±SD	% RSD
1	5 µg/ml	|-0.012|±0.0002	1.65
2	10 µg/ml	|-0.023|±0.0003	1.48
3	15 µg/ml	|-0.036|±0.0005	1.42
4	20 µg/ml	|-0.045|±0.0006	1.34
5	25 µg/ml	|-0.057|±0.0007	1.24
6	30 µg/ml	|-0.069|±0.0008	1.16

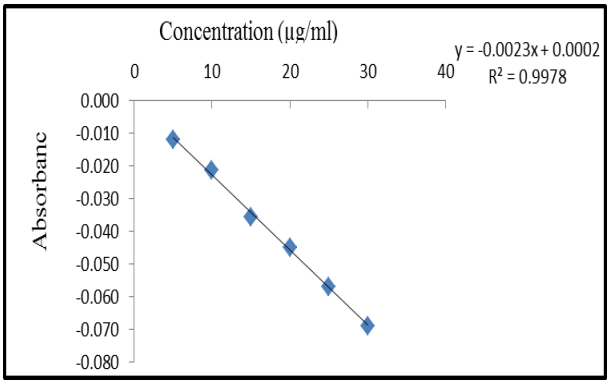

Fig 11: Calibration curve of Dapagliflozin propanediol at 288 nm.

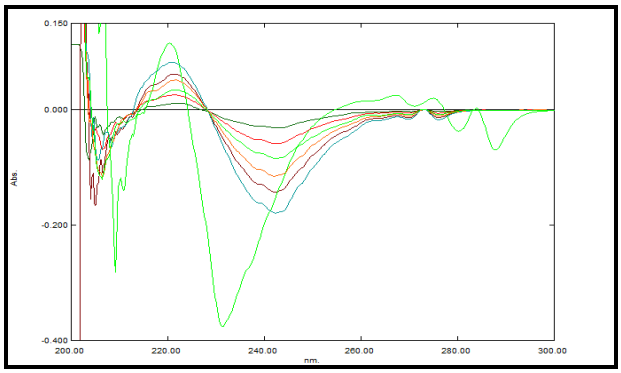

Fig 12: First order Spectra of Glimepiride in Methanol at 224 nm (5-30 µg/ml).

Table 7: Linearity data for Glimepiride.

Sr No	Concentration (µg/ml)	Mean Absorbance ±SD	% RSD
1	5 µg/ml	0.007±0.0001	1.42
2	10 µg/ml	0.022±0.0003	1.36
3	15 µg/ml	0.034±0.0004	1.17
4	20 µg/ml	0.047±0.0005	1.06
5	25 µg/ml	0.057±0.0006	1.05
6	30 µg/ml	0.068±0.0007	1.02

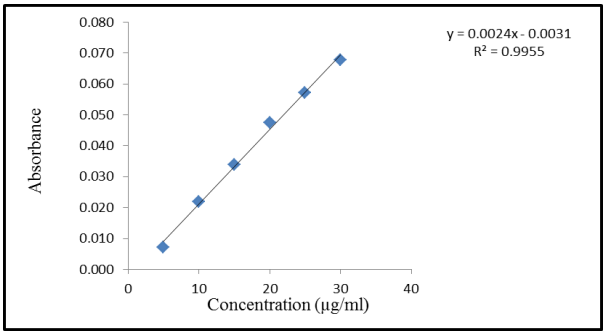

Fig 13: Calibration Curve of Glimepiride at 224 nm in UV.

➢ **Precision**

1. Intraday Precision

- The data for Intraday precision for DAPA and GLM is in range of % RSD was found to be 0.83-1.26% for DAPA at 288 nm and 0.84-1.42% for GLM at 224 nm respectively. It is shown in table 8

Table 8: Precision data for Dapagliflozin propanediol and Glimepiride (Intraday).

DRUG	CONC. (µg/ml)	Absorbance mean ± S.D. (n=3)	% RSD
		288 nm	288 nm
Dapagliflozin propanediol	5	-0.0118 ±0.00015	1.26
	10	-0.0209 ±0.00021	0.99
	15	-0.0360 ±0.00030	0.83
		224 nm	224 nm
Glimepiride	5	0.0070±0.00010	1.42
	10	0.0221±0.00026	1.17
	15	0.0341±0.00029	0.84

2. Interday Precision

- The data for Interday precision for DAPA and GLM is in range of % RSD was found to be 1.00-1.45% for DAPA at 288 nm and 1.05-1.47% for GLM at 224 nm respectively. It is shown in table 9

Table 9: Precision data for Dapagliflozin propanediol and Glimepiride(Interday).

Drug	CONC. (µg/ml)	Absorbance mean ± S.D. (n=3)	% RSD
		288 nm	288 nm
Dapagliflozin propanediol	5	-0.0118 ±0.00017	1.44
	10	-0.0208 ±0.00025	1.21
	15	-0.0357 ±0.00036	1.01
		224 nm	224 nm
Glimepiride	5	0.0068±0.00010	1.47
	10	0.0211±0.00026	1.25
	15	0.0336±0.00036	1.07

➢ **Repeatability**

- The data for repeatability for DAPA and GLM was found to be 0.84 % for DAPA at 288 nm and 0.74 % for GLM at 224 nm respectively.

Table 10: Repeatability data for Dapagliflozin propanediol and Glimepiride.

Drug	Conc. (µg/ml)	Absorbance mean± S.D. (n=6)	% RSD
		288 nm	288 nm
Dapagliflozin propanediol	10	-0.021 ±0.00018	0.84
		224 nm	224 nm
Glimepiride	10	0.022 ± 0.00016	0.74

➢ **LOD and LOQ**

- LOD value for DAPA and GLM were found to be 0.2869µg/ml and 0.054µg/ml respectively. LOQ value for DAPA and GLM were found to be 0.8695µg/ml and 0.1639µg/ml respectively.

Table 11: LOD and LOQ data table.

Parameter	Dapagliflozin propanediol	Glimepiride
Standard deviation	0.0002	0.0001
Slope	0.0023	0.0061
LOD(µg/ml)	0.2869	0.054
LOQ µg/ml)	0.8695	0.1639

➢ **Accuracy**

- Accuracy of the method was confirmed by recovery study from marketed formulation at three levels (50%, 100%, 150%) of standard addition. Percentage recovery for DAPA and GLM were found to be in the range of 98-99.5 % and 98-99%. Data indicating recovery studies of DAPA and GLM shown in table 12

Table 12: Data indicating recovery studies for Dapagliflozin propanediol and Glimepiride.

Drug	Level (%)	Amount Taken (µg/ml)	Amount Added (µg/ml)	Total Amount (µg/ml)	% recovery± S.D.(n=3)
Dapagliflozin Propanediol	50	10	5	15	98.93±0.2136
	100	10	10	20	99.00±0.1527
	150	10	15	25	99.28±0.2052
Glimepiride	50	10	5	15	98.06±0.2970
	100	10	10	20	98.75±0.1365
	150	10	15	25	98.96±0.2302

Table 13: Analysis of Synthetic Mixture.

Drug	Amount in Synthetic Mixture Taken (µg/ml)	Amount Found (µg/ml)	% Assay± S.D. (n=3)
Dapagliflozin propanediol	10	9.82	98.2±0.4041
Glimepiride	10	9.85	98.5±0.4856

Table 14: Regression analysis data and summary of validation parameters for the proposed methods

Parameter	Dapagliflozin Propanediol	Glimepiride
Wavelength (λ)	288 nm	224 nm
Beer's Law range	5-30 µg/ml	5-30 µg/ml
Regression Values:		
i. Slope	-0.0023	0.0061
ii. Intercept	0.0003	-0.0031
iii. Regression coefficient (r^2)	0.997	0.995
Accuracy (% recovery, n=3)	98-99.5	98-99
Repeatability (%RSD, n=6)	0.84	0.74
Intraday (%RSD, n=3)	0.8-1.26	0.8-1.42
Interday (%RSD, n=3)	1.01-1.44	1.07-1.47
Assay	98.2 %	98.5 %

❖ **RP-HPLC Method**

❖ **Experimental work**

❖ **Instrument and apparatus**

- A Shimadzu HPLC (LC- 2010 –CHT) Instrument [software Lab solution]
- Column- Peerless C-18 (250×4.6 mm, 5 µm)
- Digital Analytical Balance – Wensar DA 13–220 (India)
- pH meter (Thermo Electron Crop., Pune, India)
- Sonicator – Equitron (India)
- Volumetric flask – 10, 50 and 100 (Borosil)
- Pipettes – 1, 2, 5 and 10 ml (Borosil)
- Beaker – (Borosil).

➢ **Chemicals and Materials**

- Acetonitrile- Avantor Performance Material India Ltd. (HPLC grade)
- Methanol- Finar Ahmedabad. (HPLC grade)
- Water- Astron Chemical India. (HPLC grade)
- OPA (75% Ortho Phosphoric Acid) - AR Grade, Astron Chemical India.
- Dapagliflozin propanediol (Shree Parikh Trading, Ahmedabad)
- Glimepiride (West Coast, Ahmedabad)
- Hamilton syringe

➢ **Preparation of standard stock solution**

- **Dapagliflozin propanediol (100 µg/ml)**

Accurately weighed Dapagliflozin propanediol (10 mg) was transferred to a 100 ml volumetric flask, and diluted to the mark with methanol to obtain a standard stock solution (100µg/ml).

- **Glimepiride (100 µg/ml)**

Accurately weighed Glimepiride (10 mg) was transferred to a 100 ml volumetric flask, and diluted to the mark with methanol to obtain a standard stock solution (100µg/ml).

➢ **Preparation of binary mixture of Dapagliflozin propanediol and Glimepiride**

- Standard stock solution of Dapagliflozin propanediol (5 ml) and Glimepiride (5 ml) was transferred to a 50 ml volumetric flask and diluted up to the mark with ACN : Water (70:30)

❖ **Selection of Detection Wavelength**

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. Absorbance maximum was obtained was at 228 nm. So, 228 nm was selected for detection of Dapagliflozin propanediol and Glimepiride.

❖ **Mobile phase selection**

The composition and flow rate of mobile phase were changed to optimize the separation condition using combined solution. The pKa value for Dapagliflozin propanediol and Glimepiride is 12.57 and 4.75 respectively. After number of trial experiments, it was established that the mobile phase ACN: water (pH 6.0 adjusts with Ortho phosphoric acid) (70:30 % v/v) shows good peak shape and resolution.

- **Mobile phase:** ACN: water (pH 6.0 with Ortho phosphoric acid) (70:30 % v/v)
- **Preparation of 10% Ortho phosphoric acid:** 10% Ortho phosphoric acid was prepared by diluting 1.33 ml of concentrated Ortho phosphoric acid in 10 ml HPLC grade water.

❖ **Chromatographic condition**

Column: Peerless C-18 (250 mm × 4.6 mm, 5 µm)
Mobile phase: ACN: water (pH 6.0 adjusts with Ortho phosphoric acid)(70:30 % v/v)
Flow rate: 1.0 ml/min
Run time: 10 min
Detection wavelength: 228 nm
Detector: U.V Detector
Injection volume: 20 µl

❖ **Preparation of calibration curve**

Aliquots equivalent to 0.1, 0.2, 0.3, 0.4 and 0.5 ml working standard solution of Dapagliflozin propanediol and Glimepiride were transferred into a series of five 10 ml Volumetric flasks and volume was adjusted to the mark with mobile phase to get concentration 1, 2, 3, 4, and 5 µg/ml of Dapagliflozin propanediol and Glimepiride. 20 µl of each of the solution were injected into HPLC system and analyzed. Calibration curve was obtained by plotting respective peak area against concentration in µg/ml and the regression equation was computed.

❖ **Method Validation**

The developed method was validated with respect to linearity, accuracy, precision, limit of detection and limit of quantification in accordance with the ICH guideline.

➢ **Specificity**

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

➢ **Linearity & Range**

The linearity of Dapagliflozin propanediol and Glimepiride was found to be in the range of 1 – 5 µg/ml and 1 – 5 µg/ml respectively. Plot the calibration curve of Area (µV.s) vs. Concentration (µg/ml). Linearity of both the drugs was checked in term of slope, intercept and correlation coefficient.

➢ **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 may be consider at three levels: Intermediate (Intraday) precision, reproducibility (Interday precision), repeatability.

1) **Intraday Precision:** Solution containing 1, 2 and 3 µg/ml of Dapagliflozin propanediol and 1, 2 and 3 µg/ml of Glimepiride were analyzed three times on the same day and %RSD was calculated.
2) **Interday Precision:** Solutions containing 1, 2 and 3 µg/ml of Dapagliflozin propanediol and 1, 2, and 3 of Glimepiride were analyzed three different successive days and %RSD was calculated.
3) **Repeatability:** Solutions containing 2 µg/ml of Dapagliflozin propanediol and 2 µg/ml of Glimepiride were analyzed for six times and %RSD was calculated. %RSD was not more than 2%.

➢ **Limit of Detection (LOD)**

Limit of Detection can be calculated using following equation as per ICH guidelines.

$$LOD = 3.3 \times (\sigma/S)$$

Where, σ = standard deviation of the Y intercept of calibration curve
S = Mean slope of the corresponding calibration curve.

➢ **Limit of Quantification (LOQ)**

Limit of Quantification can be calculated using following equation as per ICH guidelines.

$$LOQ = 10 \times (\sigma/S)$$

Where, σ = standard deviation of the Y intercept of calibration curve
S = Mean slope of the corresponding calibration curve.

➢ **Accuracy**

The Accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Accuracy of the developed method was confirmed by doing recovery study as per ICH guideline at three different concentration levels 50%, 100%, 150% and the values were measured at all wavelengths for 2 µg/ml of Dapagliflozin propanediol and 2 µg/ml of Glimepiride. This performance was done in triplicate.

The amount of Dapagliflozin propanediol and Glimepiride were calculated at each level % recoveries were calculated by measuring the peak area and fitting the values in equation.

❖ **Analysis of Synthetic Mixture**

➢ **Preparation of Synthetic Mixture of Dapagliflozin propanediol and Glimepiride**

- The Synthetic Mixture of Dapagliflozin propanediol and Glimepiride was prepared in ratio of 1:1
- excipients, Microcrystalline Cellulose, starch, Magnesium Stearate, lactose, colloidal silicon dioxide along with the drug Dapagliflozin propanediol 10 mg and Glimepiride 10 mg.
- Accurately weighed equivalently weight of Dapagliflozin propanediol (10 mg) and Glimepiride (10 mg) which transferred in 100 ml volumetric flask and make up half mark with Methanol. This solution was Sonicated till the drug dissolves and was made up to mark with Methanol. Then this solution was filtered through Whatmann filter paper. So, obtained concentration of Dapagliflozin propanediol is 100 µg/ml and Glimepiride is 100 µg/ml.

➢ **Preparation of Working Solution**

➢ From the stock solution of synthetic mixture (100 µg/ml Dapagliflozin propanediol and 100 µg/ml Glimepiride), pipette out 0.2 ml and transferred into volumetric flask of 10 ml and make up the volume with Methanol, to get the concentration of 2 µg/ml and 2 µg/ml for Dapagliflozin propanediol and Glimepiride respectively. Different ml of standard stock solutions of Dapagliflozin propanediol and Glimepiride was pipette out to get the concentration of 50%, 100% and 150%

Table 15: Amount of Drug taken in 10 ml of volumetric flask for Dapagliflozin propanediol

% Level of Recovery	Amount of Drug Taken (µg/ml)	Amount of Drug Spiked (µg/ml)	Total Concentration (µg/ml)
50	2	1	3
100	2	2	4
150	2	3	5

- Each Flask was made up to 10 ml with methanol. Each process was carried out 3 times.

Table 16: Amount of Drug taken in 10 ml of volumetric flask for Glimepiride.

% Level of Recovery	Amount of Drug Taken (µg/ml)	Amount of Drug Spiked (µg/ml)	Total Concentration (µg/ml)
50	2	1	3
100	2	2	4
150	2	3	5

- Each Flask was made up to 10 ml with methanol. Each process was carried out 3 times.

➢ **Robustness**

Change following parameters, one by one and observe their effect on system suitability test and assay.

- Change the minor components in the mobile phase.
- Change in detection wavelength 2 nm (226 nm and 230 nm).

➢ **System suitability tests**

System suitability tests is an integral part of liquid chromatography. They are used to verify that resolution and reproducibility of chromatography system are adequate for the analysis to be done. The tests include Resolution (R), Column efficiency (N), Tailing factor (T) and Precision of replicate injection.

❖ **RESULT AND DISCUSSION**

❖ **Selection of elution mode**

- Reverse phase chromatography was chosen because of its recommended use for ionic and moderate to non-polar compounds. Reverse phase chromatography is not only simple, convenient but also better performance in terms of efficiency, stability and reproducibility. C18 column was selected because it is least polar compare to C4 and C8 columns.
- C18 column allows eluting polar compounds more quickly compare to non-polar compounds. In addition to this, UV detector is used which allows easy detection of the compounds in UV transperant organic solvents. A 250 mm × 4.6 mm column of 5 µm particles packing was for separation of Dapagliflozin propanediol and Glimepiride. Isocratic mode column stability. This configuration provides a large number of theoretical plate's values for most separation.

➢ **Selection of Detection Wavelength**

- The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. Absorbance maximum was obtained was at 228 nm. So, 228 nm was selected for detection of Dapagliflozin propanediol and Glimepiride.

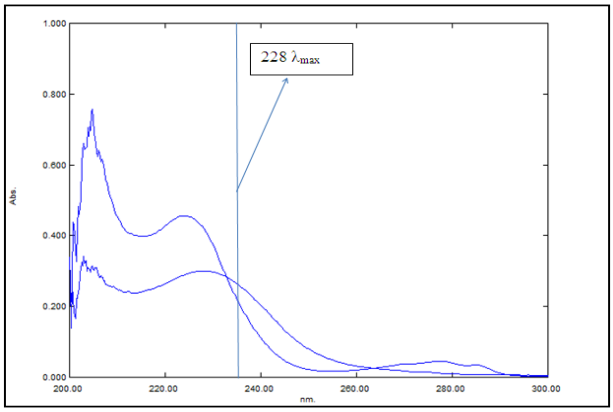

Figure 15: zero order spectra of Dapagliflozin propanediol (10 µg/ml) and Glimepiride (10 µg/ml) in methanol.

➢ **Optimization of Chromatographic conditions**

Various mobile phases, such as Methanol: Water, Acetonitrile: Water, phosphate buffer: Acetonitrile, Phosphate Buffer : Methanol in different proportion was tried. The combination of Acetonitrile: Water (pH 6) (70:30 v/v) provided optimum polarity for proper migration, sepration and resolution of Dapagliflozin propanediol and Glimepiride. Under these conditions, the eluted peaks were well defined, resolved and free from tailing. The elution order was Dapagliflozin propanediol (Rt =3.100) and Glimepiride (Rt =6.760) at a flow rate of 1 ml /min.

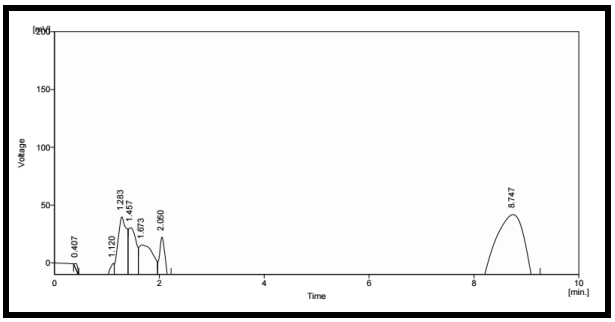

Figure: Trial 1 Chromatogram of Dapagliflozin propanediol (3µg/ml) and Glimepiride (3 µg/ml) in Methanol: Water (pH 6.5) (80: 20 %v/v).

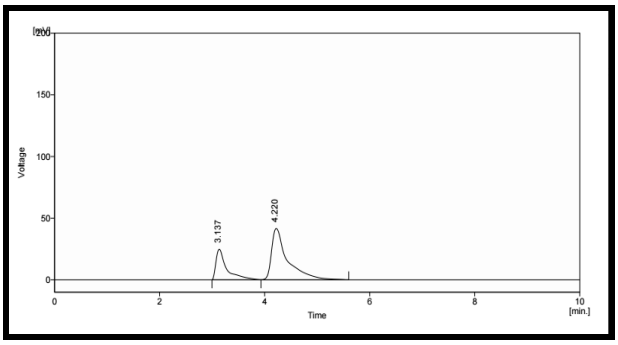

Figure: Trial 2 Chromatogram of Dapagliflozin propanediol (3µg/ml) and Glimepiride (3µg/ml) in ACN: Water (pH 6.5) (80: 20 %v/v).

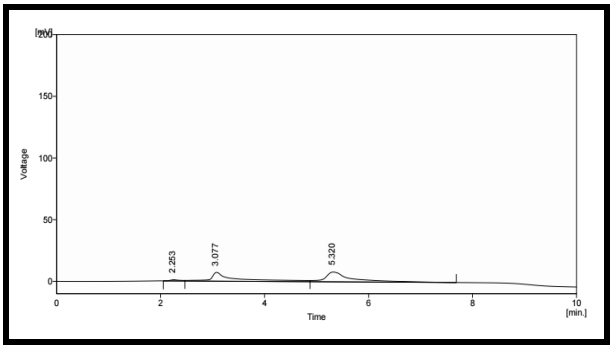

Figure: Trial 3 Chromatogram of Dapagliflozin propanediol (3µg/ml) and Glimepiride (3µg/ml) in ACN: Water (pH 6.5) (70: 30 %v/v).

Table 16: Mobile phase optimization trial for Dapagliflozin propanediol and Glimepiride.

Trial	Mobile Phase	Ratio (v/v)	Remark
1	Methanol: Water	80 : 20	Peak was not obtained
2	ACN: Water (pH= 6.5)	80 : 20	Resolution was not proper
3	ACN : Water (pH= 6.5)	70 : 30	Tailing was obtained
4	ACN: Water (pH= 6.0)	70:30	Good resolution, sharp peak and peak good resolution

➢ **Method validation**

➢ **Specificity**

It was prove by comparing the chromatogram of mobile phase, standard solution and test preparation solution to show that there was no peak of mobile phase and no any interference of excipients with the peak of Dapagliflozin propanediol and Glimepiride as shown in figure 5.2, 5.3, 5.4, and 5.5.

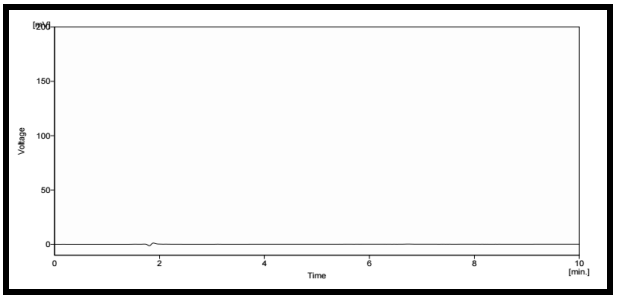

Figure 17: Chromatogram of Blank.

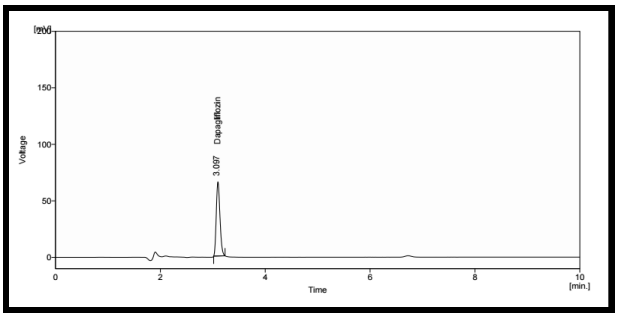

Figure 18: Chromatogram of Dapagliflozin propanediol (3µg/ml) in ACN: Water (pH 6.0) (70: 30 %v/v).

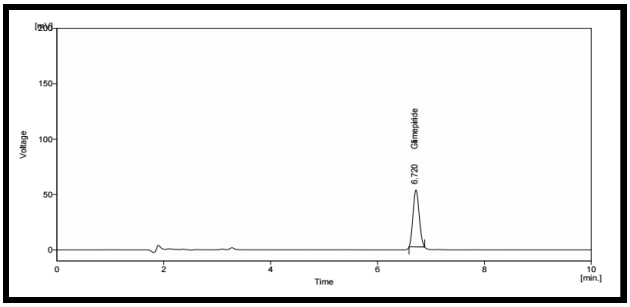

Figure 19: Chromatogram of Glimepiride (3µg/ml) in ACN: Water (pH 6.0) (70: 30 %v/v).

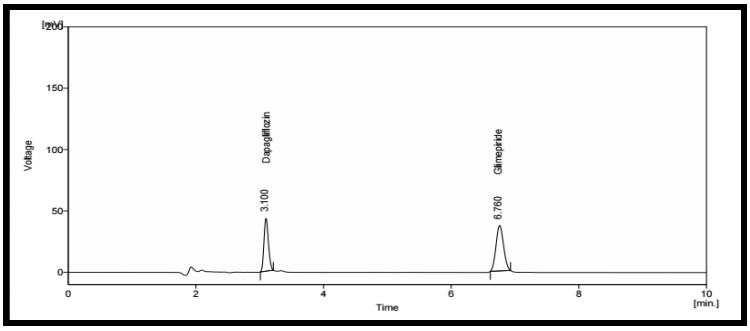

Figure 20: Chromatogram of Dapagliflozin propanediol (2µg/ml) and Glimepiride (2µg/ml) in ACN: Water (pH 6) (70: 30 %v/v).

- **System suitability parameters**

The resolution, asymmetry factor, tailing factor and number of theoretical plates are shown in table 5.4. The values obtained demonstrated the suitability of the system for the analysis of these drugs in combination.

Table 17: System suitability parameter.

Parameters	Retention Time	Tailing Factor	Number of Theoretical Plate	Resolution
Dapagliflozin propanediol	3.100	1.316	9058	-
Glimepiride	6.760	1.176	13559	20.24

- **Linearity**

The linearity of DAPA and GLM was found to be in the range of 1-5 µg/ml. Linearity data for DAPA and GLM are depicted in Table 5.5.

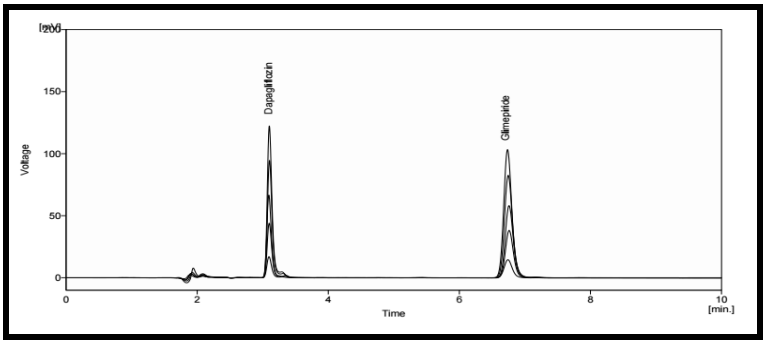

Figure 21: Overlay chromatogram of Dapagliflozin propanediol and glimepiride (1-5 µg/ml).

Table 18: Calibration data for DAPA (1-5 µg/ml) and GLM (1-5 µg/ml).

Sr. No	Concentration (µg/ml)		Peak area (µV*sec) (Mean ± S.D.) N=5		%RSD	
	DAPA	GLM	DAPA	GLM	DAPA	GLM
1	1	1	65585±131.67	103340.4±799.30	0.20	0.77
2	2	2	210721.2±902.27	324154.2±2589.52	0.42	0.79
3	3	3	406337.8±1468.41	515267.4±3652.06	0.36	0.70
4	4	4	584341.4±2301.14	685124.6±3405.93	0.39	0.49
5	5	5	785400.2±3398.42	936292.2±3525.84	0.43	0.37

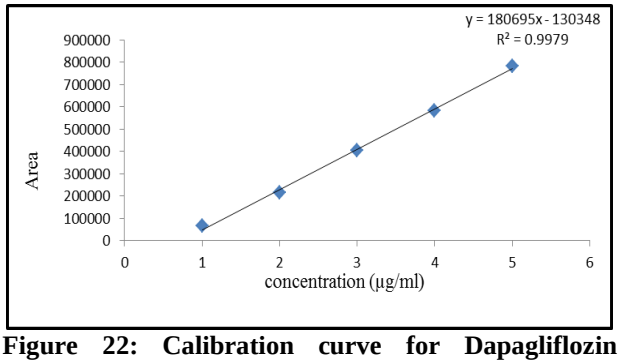

Figure 22: Calibration curve for Dapagliflozin propanediol (1-5 µg/ml).

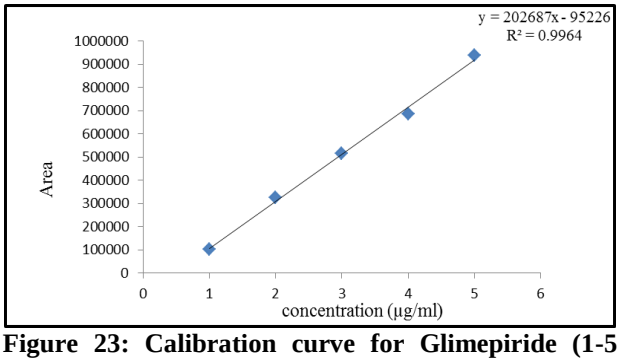

Figure 23: Calibration curve for Glimepiride (1-5 µg/ml).

- **Precision**
- **Intraday Precision**

- The data for Intraday precision for DAPA and GLM is in range of % RSD was found to be 0.10-0.50% for DAPA at 228 nm and 0.35-0.65% for GLM at 228 nm respectively. It is shown in table 5.6

Table 5.6: Precision data for Dapagliflozin propanediol and Glimepiride (Intraday).

DRUG	CONC. (µg/ml)	Mean Peak Area (µV*sec) ± SD(n = 3)	% RSD
Dapagliflozin propanediol	1	65561.67±179.53	0.27
	2	216662±322.40	0.14
	3	406013±1966.166	0.48
Glimepiride	1	103658.3±398.33	0.38
	2	322228±1209.74	0.37
	3	515018.3±3215.33	0.62

- **Interday Precision**

- The data for Interday precision for DAPA and GLM is in range of % RSD was found to be 0.30-0.95% for DAPA at 228 nm and 0.80-1.05% for GLM at 228 nm respectively. It is shown in table 5.7

Table 5.7: Precision data for Dapagliflozin propanediol and Glimepiride (Interday).

DRUG	CONC. (µg/ml)	Mean Peak Area (µV*sec) ± SD(n = 3)	% RSD
Dapagliflozin propanediol	1	65421.67±220.01	0.33
	2	217341.3±1088.047	0.50
	3	403976.7±3733.64	0.92
Glimepiride	1	103527±1061.95	1.02
	2	326195.7±3164.94	0.97
	3	514657.3±4359.69	0.84

- **Repeatability**

The data for repeatability for DAPA and GLM was found to be 0.55 % and 0.47 % respectively.

Table 5.8 Repeatability data for Dapagliflozin propanediol and Glimepiride.

Drug	Conc. (µg/ml)	Mean Peak Area (µV*sec) ± SD(n = 3)	% RSD
Dapagliflozin propanediol	2	217053.3±1207.31	0.55
Glimepiride	2	323823.8±1548.81	0.47

- **Accuracy**

Accuracy of the method was confirmed by recovery study from synthetic mixture at three levels (50%, 100%, 150%) of standard addition. Percentage recovery for DAPA and GLM were found to be in the range of 99-99.50 % and 99.0-99.50%. Data indicating recovery studies of DAPA and GLM shown in table 5.9

Table 5.9: Accuracy study data.

Drug	% Level of Recovery	Amount of drug taken (µg/ml)	Amount of drug added (µg/ml)	Total amount taken (µg/ml)	% Recovery ± S.D. (n=3)
Dapagliflozin propanediol	50	2	1	3	99.33±0.41
	100	2	2	4	99.20±0.26
	150	2	3	5	99.30±0.19
Glimepiride	50	2	1	3	99.06±0.13
	100	2	2	4	99.08±0.15
	150	2	3	5	99.41±0.24

- **LOD and LOQ**

LOD value for DAPA and GLM were found to be 0.0024µg/ml and 0.013µg/ml respectively. LOQ value for DAPA and GLM were found to be 0.0072µg/ml and 0.039µg/ml respectively.

Table 5.10: LOD and LOQ data table.

Parameter	Dapagliflozin propanediol	Glimepiride
Standard deviation	131.67	799.30
Slope	180695	202687
LOD(µg/ml)	0.0024	0.013
LOQ µg/ml)	0.0072	0.039

➢ **Assay**

Applicability of the proposed method was tested by analyzing the synthetic mixture. The % Assay for Dapagliflozin propanediol and Glimepiride were found to be 99.26% and 99.2% respectively. The results are shown in table 5.11.

Table 5.11: Application of HPLC method to Synthetic mixture.

Drug	Amount taken (µg/ml)	Amount found (µg/ml)	% Assay ± S.D. (n=3)
Dapagliflozin propanediol	**2**	1.98	99.26±0.321
Glimepiride	**2**	1.97	99.2±0.200

➢ **Robustness**

Change following parameters, one by one and observe their effect on system suitability test and assay.
Change the minor components in the mobile phase.
Change in detection wavelength 2 nm (226 nm and 230 nm).

Table 5.12: Robustness data of Dapagliflozin propanediol and Glimepiride.

Condition	Variation	Dapagliflozin propanediol	Glimepiride
		% Assay ± SD (n=3)	% Assay ± SD (n=3)
Standard		**99.26±0.321**	**99.2±0.200**
Detection wavelength (228 ±2nm)	**226 nm**	98.17±0.5571	98.73±0.3659
	230 nm	99.18±0.3176	98.85±0.5294
Change in Mobile phase	**68:32**	99.12±0.5497	97.73±0.9864
	72:28	99.23±0.4271	98.80±0.7347

➢ **Summary**

Table 5.13: Summary of Validation Parameters.

Sr. No.	Parameters	Dapagliflozin propanediol	Glimepiride
1.	Beer's Law Limit (µg/ml)	1-5	1-5
2.	Regression equation (y= mx + c)	Y=180695x-130348	Y=202687x-95226
3.	Correlation Coefficient (r^2)	0.997	0.996
4.	Repeatability (% RSD, n=6)	0.55	0.47
5.	Intraday Precision (% RSD, n=3)	0.14-0.48	0.35-0.65
6.	Interday Precision (% RSD, n=3)	0.30-0.95	0.80-1.10
7.	% Recovery	99.20-99.35	99.00-99.41
8.	LOD (µg/ml)	0.0024	0.013
9.	LOQ (µg/ml)	0.0072	0.039
10.	Assay (%)	99.26	99.2

CONCLUSION

Simple, rapid, accurate and precise RP-HPLC and UV spectrophotometric methods have been developed and validated for the routine analysis of Dapagliflozin propanediol and Glimepiride in synthetic mixture. Both methods are suitable for the simultaneous determination of Dapagliflozin propanediol and Glimepiride in multicomponent formulation without interference of each other. The amount found from the proposed methods were found in good agreement with the label claim of the formulation. Also the value of standard deviation and coefficient of variation calculated were satisfactorily low, indicating the suitability of the proposed methods for the routine estimation of combination dosage forms.

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