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Bioanalytical Stability Indicating Method Development and Validation for the Estimation of Anti Diabetic Drugs in Human Plasma by Using RP –HPLC Method.

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KEYWORDS

Bio-analytical method, RP-HPLC, DAD, Validation, Metformin, Dapagliflozin

ABSTRACT:

A reverse phase HPLC has been developed and validated for estimation of Metformin and Dapagliflozin in Human plasma. In this method, a reverse phase column Agilent – C18 Plasma 5 µm (4.6× 250 mm) as a Stationary phase, with a Mobile Phase of Methanol and 0.05% ortho phosphoric acid in water (pH 2.5) in the ratio of (50%:50%) at 0.8 mL/min flow rate was used with a DAD detection was carried out at 233nm. The volume injected was 20 µL. The described HPLC method was linear over a concentration range of 100 to 500 µg/ml for Metformin and 2 to 10µg/ml for Dapagliflozin. The Metformin showed retention time 2.222 min &Dapagliflozin showed retention time 4.429 min respectively. The Accuracy and Precision, recovery, selectivity for metformin was 100-500 µg/ml respectively& for Dapagliflozin 2 to 10 µg/ml. The stability of the drug spiked human during three freeze thaw cycles were stable in plasma for about one month when stored at frozen state.

Introduction:

Globally, diabetes mellitus (DM) affects over 400 million people and is a significant public health concern. This metabolic disorder progressively leads to chronic microvascular, macrovascular and neuropathic life threatening complications.[1] A propensity for sedentary living could be the main cause of the ongoing global increase in the number of diabetic patients, which is predicted to reach 366 million in 2030 among the elderly (>65 years) [2]. Nephropathy, neuropathy, renal and cardiovascular problems, retinopathy, food-related disorders, and other issues are among the many complications linked to diabetes mellitus. There are two types of DM: type 1 and type 2. Type 2 diabetes is caused by impairment of pancreatic beta cells, which impairs the person's ability to use insulin. Type 1 diabetes is an autoimmune disease that affects pancreatic cells, reducing or impairing the production of insulin. [3]

Therapeutic approaches in non-insulin treatment for type 2 diabetes mellitus.

A number of non-insulin based oral therapies have emerged for the treatment of type 2 DM. These are categorized under the following sub-headings: [4]

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1 Insulin Secretagogues

2 Biguanides

3 Insulin Sensitizers

4 Alpha Glucosidase Inhibitors

5 Incretin mimetics

6 Amylin antagonists

7 SGLT2 inhibitors.

In this article Bigunide (Metformin) and SGL2 Inhibitors (Dapagliflozin) are studied. Biguanides are oral medications that help patients with mild to moderately severe type 2 diabetes overcome their insulin resistance. They are particularly useful for obese patients who do not respond well to diet and exercise therapy [5-6]

Biguanides work in two ways: first, they increase the uptake and utilization of glucose in peripheral muscle by increasing the sensitivity of muscle and fat cells to available insulin; second, they inhibit the production of glucose in the liver by stopping it from producing too much glucose [7]. A novel class of diabetic drugs called sodium-glucose co-transporter 2 (SGLT2) inhibitors is used to treat type 2 diabetes. In humans, the protein known as SGLT2 helps the kidneys absorb glucose once more. By preventing the kidneys from reabsorbing glucose and increasing glucose excretion, SGLT2 inhibitors minimize blood glucose levels. [8]

Patients with type 2 diabetes mellitus can benefit from the combination of Dapagliflozin and Metformin as a therapeutic option (T2DM). The fixed-dose combination of Dapagliflozin and Metformin, along with their unique combined mechanism of action, favorable efficacy, and safety profile, lend support to its use as a treatment option for patients with type 2 diabetes [9]

Description of Dapagliflozin

In patients with type 2 diabetes, Dapagliflozin is used to lower blood sugar levels and reduces the risk of kidney damage, blindness, and limb loss. Additionally prevented are nerve problems and sexual function. This medication is also prescribed to patients with type 2 diabetes and heart disease in order to lower their risk of developing heart failure. [10-15]

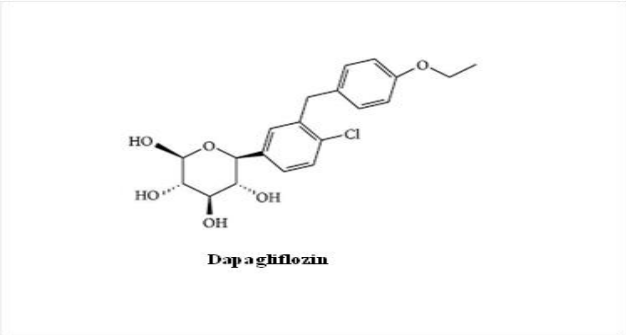

Figure 1: Chemical structure of Dapaglioflozin

Chemically speaking, Dapagliflozin is known as (1s)-1, 5-anhydro1-C-[4-chloro-3-[(4-ethoxyphenyl) methyl] phenyl]-Dglucitol. Its molecular weight is 408.98 and its molecular formula is C24H33ClO8. (16), Its Solubility in Methanol, Water, and Acetonitrile [17-19]

Description of Metformin Hydrochloride

Metformin

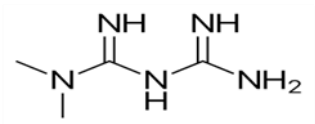

Figure 2: Chemical structure of Metformin

The chemical formula for metformin hydrochloride is 3-(diaminomethylidene)-1,1-dimethylguanidine; hydrochloride. Its molecular weight is 129.167 and Its Molecular formula is$C_4H_{11}N_5$ [20]

This medication is used to treat diabetes type 2. Bigunide used to treat hyperglycemia is metformin. It raises body tissues' sensitivity to insulin while decreasing the liver's production of glucose.

Importance of the Combination Dosage Form

A combination is advised for type 2 diabetics in order to enhance glycemic control. Extended-release tablets are one of the forms they come in.

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Dapagliflozin stops the kidneys from absorbing glucose. This aids in lowering blood sugar levels. Metformin hydrochloride works by decreasing the amount of sugar that is released from the liver and absorbed from the stomach. This medication lowers the risk of heart failure in patients with type 2 diabetes and heart disease.[21]

Bioanalysis:

Drug concentrations, their metabolites, and/or endogenous substances can all be found in biological matrices like blood plasma, serum, cerebrospinal fluid, urine, and saliva by using a technique called bioanalysis. Drugs and their metabolites in physiological matrices are frequently quantified using bioanalytical methods, which can also be used in studies in non-human pharmacology and toxicology as well as human clinical pharmacology. The assessment and interpretation of bioequivalence, pharmacokinetics, and toxic kinetic studies are greatly influenced by the bioanalytical method used for the quantitative determination of pharmaceuticals and their metabolites in biological fluids. [22-24]

Extraction Procedure for Biological sample

Various kinds of extraction techniques are

- Injection after dilution.
- Solid Phase extraction (offline or online)
- Protein precipitation,
- Filtration,
- liquid-liquid extraction,
- Solid-supported liquid-liquid extraction,
- Equilibrium dialysis, Ultra filtration,
- Restricted access media,
- Monolithic columns,
- Immunoaffinity extraction

These are some of the methods that can be used to remove proteins from blood plasma. But most widely used methods that have been used to extract analyte from biological matrix: (a) liquid-liquid extraction (LLE), (b) solid-phase extraction (SPE), and (c) precipitation of plasma proteins.[25-27]

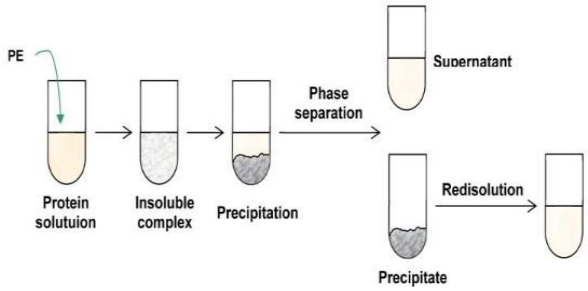

Fig 3: Schematic diagram of protein precipitation technique.

Protein Precipitation (28-29)

An extremely basic method for removing the analyte from blood or plasma is protein precipitation. The analyte must be freely soluble in the reconstituting solvent in order to be used in this technique.Using acids (trichloroacetic acid and perchloric acid), organic solvents (methanol, ethanol, acetone, and acetonitrile), or salts (ammonium sulphate), the sample is prepared by protein precipitation. In this technique, the sample is centrifuged after precipitation, and the analyte is placed in the supernatant. Methanol is the recommended solvent because it yields that can be injected directly. It is possible to extract both hydrophilic and hydrophobic substances using PP. The drawback is that PP could clog the column.

BIOANALYTICAL METHOD VALIDATION (BMV) [30]

A biological matrix for a chemical compound is collected, processed, stored, and analyzed using a set of steps known as a bioanalytical method. The process of determining whether a quantitative analytical method is appropriate for use in biomedical applications is known as bioanalytical method validation, or BMV. The processes that show that a specific technique for quantitatively measuring analytes in a given biological matrix—such as blood, plasma, serum, or urine—is dependable and repeatable for the intended use are collectively referred to as method validation. Validation entails proving that the method's performance characteristics are appropriate and dependable for the targeted analytical applications through the use of particular laboratory investigations.

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Validation Parameters [31]

The basic parameters for the validation of a chemical assay comprises of all criteria determining data quality such as selectivity, accuracy, precision, recovery, linearity, , limit of detection (LOD), lower limit of quantification (LLOQ), stability, reproducibility, and ruggedness.

There is no RP-HPLC methods reported for estimation of Dapagliflozin and Metformin combination spiked in human plasma. The objective of the present study was to develop and validate a new RP-HPLC Bioanalytical method for the simultaneous estimation of Dapagliflozin and Metformin in bulk, dosage form and to evaluate its application in pharmacokinetic studies for estimation of Dapagliflozin and Metformin.

Material and method:

Procured pure standards of Metformin (MET) and Dapagliflozin (DAPA) from R.S I T C Jalgaon. Purchased a tablet formulation known as Xigduo XR, which contained 500 mg of metformin.

and 10 mg of Dapagliflozin from pharmacy shop sold by it. The following products were bought from Merck Ltd methanol (HPLC grade), acetonitrile (HPLC grade), water (HPLC grade), orthophosphoric acid (AR grade), and triethylamine (AR grade).

Instrumentation:

Chromatographic measurements were made on AGILET (1100) HPLC having detector (G-13148 with DAD source in the range 233 nm with double reciprocating plunger pump with constant flow and pressure delivery. The mobile phase was degassed by using Ultrasonicator (3.5L100) Ultrasonics electronic instrument Ultra sonic bath. The UV spectrum was recorded using a UV-Visible spectrophotometer (Analytical Technologies Limited, Japan (Model UV 2080) – software UV analyst.

Mobile phase selection:

The main requirement of the mobile phase is that it has to dissolve the analytes up to the concentration suitable for detection. The mobile phase absorbance should usually be less than 0.5 at the wavelength used for detection. When the absorbance of the mobile phase exceeds a value of about 1.0 the detector may become unusable. Hence the mobile phase suitable for samples is selected by performing trials with different ratios of the mobile phase.

Chromatographic condition:

HPLC: Agilet(1100)

Software: Chemstation

Stationary phase: C18 column (Agilent)

Mobile phase: Solvent A – Methanol Solvent B – 0.05%OPA

Solvent ratio: 50: 50 (A: B)

Detection Wavelength: 233 nm

Flow rate: .0.8 ml/min

Temperature: Ambient

Sample size: 20 µl

Preparation of Mobile phase:

50.0 % of methanol and 50.0 % of 0.05%Orthophosperic acid (ph 2.5) were combined to create the mobile phase. After passing through a 0.42 µ membrane filter, this mobile phase underwent 15 minutes of ultrasonication.

Prepration of Sample Solution:

Transferred about 250 mg of Metformin (Met) and 5 mg of Dapagliflozin (Dapa) standard into a 25 mL volumetric flask, added approximately 10 mL of diluent, shaken to dissolve, and volume brought up to the mark with diluent. (Met and Dapa concentrations are 10,000µg/mL and 200µg/mL, respectively.) The solutions were brought up to volume with mobile phase using an A-grade bulb pipette into 10 ml volumetric flasks, giving final concentrations of 100-500µg/mL for Met and 2-10µg/mL for Dapa.

Preparation of plasma sample:

At the time of analysis, the samples were removed from the deep freezer and kept in the room temperature and allowed to thaw. 1.0 mL of sample was pipetted into 10 ml centrifuge tube with this 250mg of Metformin

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solution (10,000 µg/ml) and 5 mg of Dapagliflozin solution (200µg/mL) was added. The resulting solution was vortexed for 10 minutes and centrifuged at 5000 r/min for 10 min. 5000 RPM for effective phase separation. organic layer was pipette out into a separate tube and evaporated to dryness. The residue was then reconstituted with 10mL mobile phase and subjected to chromatographic analysis.

Method Development:

The mobile phase consisting of methanol and 0.05% Orthophosperic acid in varying proportions and change in pH was tried and finally ratio of 50:50 (pH-2.5 adjusted with orthophosphoric acid) was selected because it was found to give good separation for the peaks of Dapagliflozin (Rt-4.429 min) and metformin (Rt-2.222 min) respectively as shown in the **figure 7.** In addition to this, UV spectra of individual drugs were recorded at the wavelength range from 200 to 400 nm and the response for optimization was compared. The choice of wavelength 233 nm was considered satisfactory, permitting the detection of drugs with adequate.

Result and Discussion:

Method Validation:

The method was validated in accordance with USFDA guidelines and EMEA guidelines.[32]

A Bioanalytical RP-HPLC method was developed for the Dapagliflozin and metformin. The chromatographic conditions were stabilized in order to provide a good performance of the assay. The standard solutions were prepared, and chromatograms were recorded. The study proposes a method for the determination of Dapagliflozin and metformin combination in human plasma by using RP-HPLC.

Selection of wavelength for 20 µg/mL Dapagliflozin in Methanol

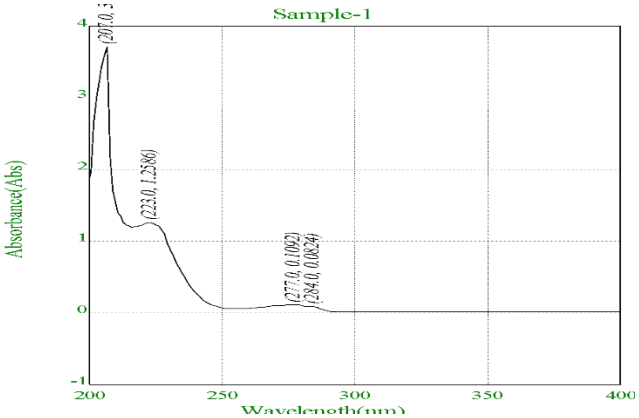

Figure 4: Uv spectrum of Dapagliflozin

Selection of wavelength for 20 µg/mL Metformin in Methanol

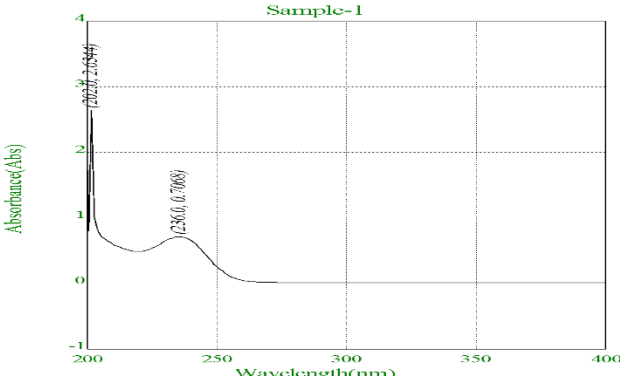

Figure 5: Uv spectrum of Metformin

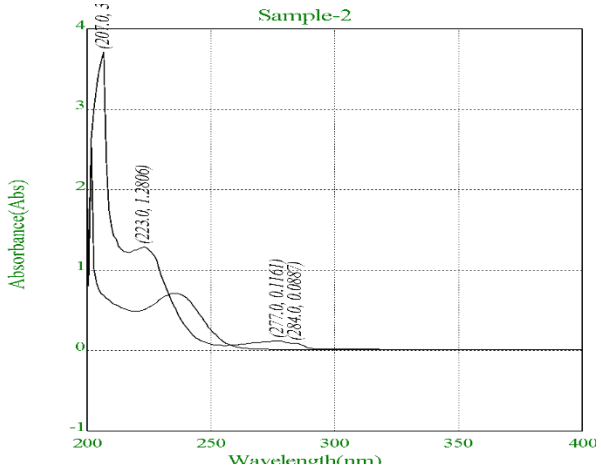

Figure 6: IsosbesticPoint of Combination drug at 233 nm

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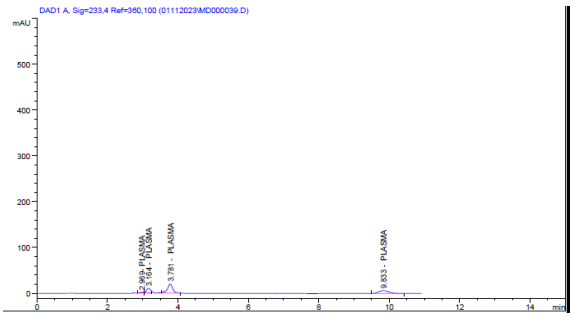

Figure 7 :Representative Chromatogram of Blank Plasma

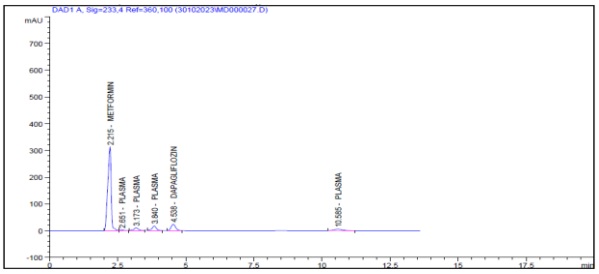

Figure 8 : Representative Chromatogram of Met and Dapa with Plasma

1)1. Specificity (selectivity)

The ability of an analytical technique to identify and measure the analyte in the presence of other sample constituents is known as selectivity. The specificity of method was performed by comparing the chromatogram of blank, standard and sample. The retention time found is stated below.

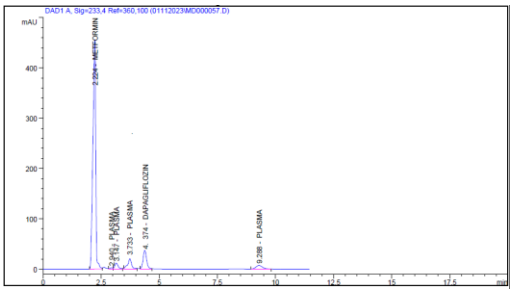

Figure 9: Specificity Chromatogram of Met and Dapa with Plasma.

Table no: 1 Specificity result Metformin and Dapagliflozin

Sr No	Solution	Area	%RSD	Retention time
1	Blank	0	0	0
2	400µg/mL Metformin	3814.70	0.148	2.224
3	8µg/mL Dapagliflozin	381.30	0.227	4.374

2. Accuracy:

Accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte.

Accuracy result for Metformin

Accuracy was computed by recoveries studies. The mean percentage recoveries values for three levels were found to be between 100.5%for Metformin respectively. The percentage of recoveries values within the limits, indicating the method developed was accurate.

Table 2: Accuracy result for Metformin

% Cons at specified level	Area	Amount added(mg)	Amount Founded (mg)	% Recovery
100 µg/mL	1762.25	80	181.73	102.16
100 µg/mL	1913.125	100	198.41	98.41
100 µg/mL	2121.246	120	221.11	100.93

***Average of three determinations**

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Accuracy Result for Dapagliflozin

The mean percentage recoveries values for three levels were found to be between 101.06%for Dapagliflozin respectively. The percentage of recoveries values within the limits, indicating the method developed was accurate.

Table no:3 Accuracy Result for Dapagliflozin

% Cons at specified level	Area	Amount added(mg)	Amount Founded (mg)	% Recovery
2 µg/mL	181.2367	1.6	3.63	101.96
2 µg/mL	200.4676	2	4.04	102.18
2 µg/mL	214.4713	2.4	4.38	99.06

***Average of three determinations**

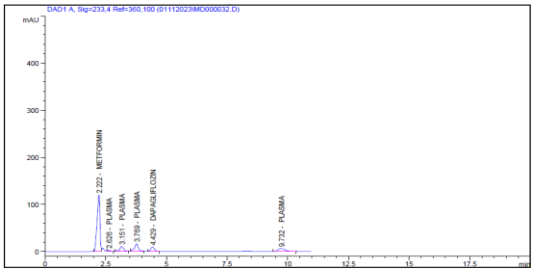

Figure 10 : Representative Accuracy Chromatogram of Met and Dapa with Plasma.

3**. Precision**: Precision of an analytical method describes the closeness of individual measures of an analyte, when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix.

The percentage RSD of system, method, and intermediate precision study was well within the limits (<2%), indicate that the method was precise.

Table no: 4 Precision Result for Metformin

Injection	Area of Metformin	SD	%RSD
300 µg/mL	2919.7531	2.41	0.08
400 µg/mL	3810.1047	1.06	0.03
500 µg/mL	4657.9594	2.94	0.06
Interday	**Area of Metformin**	**SD**	**%RSD**
300 µg/mL	2917.8341	3.86	0.13
400 µg/mL	3837.0129	0.19	0.00
500 µg/mL	4690.4526	0.34	0.01

*****Average of three determinations, %RSD: Percentage relative standard deviation

Table no: 5 Precession result for Dapagliflozin

Injection	Area of Dapagliflozin	SD	%RSD
6 µg/mL	293.03735	1.83	0.62
8 µg/mL	381.4121	1.25	0.33
10 µg/mL	470.2492	1.21	0.26

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Interday	Area of Dapagliflozin	SD	%RSD
6 µg/mL	295.5738	0.58	0.20
8 µg/mL	385.6876	0.52	0.14
10 µg/mL	475.193	0.56	0.12

*Average of three determinations, %RSD: Percentage relative standard deviation

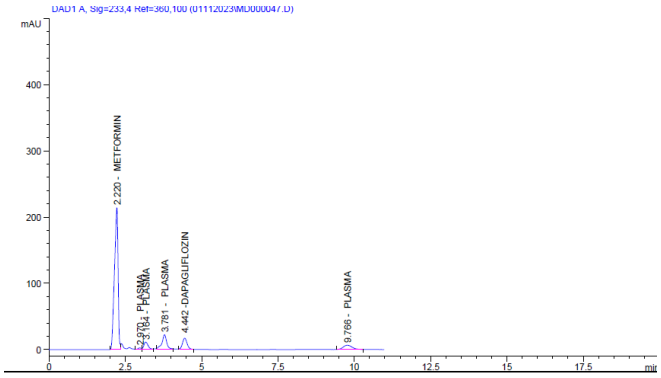

Figure 11: Representative Precision Chromatogram of Met and Dapa with Plasma.

4. **Repatability for Metformin**

Table no :6 Repatability of Metformin

Conc	Area of Metformin	SD	%RSD
500 µg/mL	4662.4192	0.61	0.01

Repatability of Dapagliflozin

Table no: 7 Repatability of Dapagliflozin

Conc	Area of Metformin	SD	%RSD
10 µg/mL	470.8851	4.01	0.85

5. Linearity

a) Metformin

Linearity was established by the least-squares linear regression analysis of the calibration data. Calibration plots were linear over the concentration range of100-500 µg/ml for metformin. Peak areas were plotted against the respective concentrations and linear regression analysis performed on the resulting curves. The linear curve of metformin was shown in **Figure 8** respectively. The linear regression equation obtained was Y=9.12x+104.2 for Metformin with correlation coefficient 0.9992 respectively. The results of linearity are shown in Table 8.

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Table no: 8 Linearity result for Metformin

Sr No	Linearity Level	Concentration	Area
1	I	100 µg/mL	986.7148
2	II	200 µg/mL	1906.319
3	III	300 µg/mL	2896.925
4	IV	400 µg/mL	3802.298
5	V	500 µg/mL	4615.358
Correlation coefficient	R^2= 0.9992		

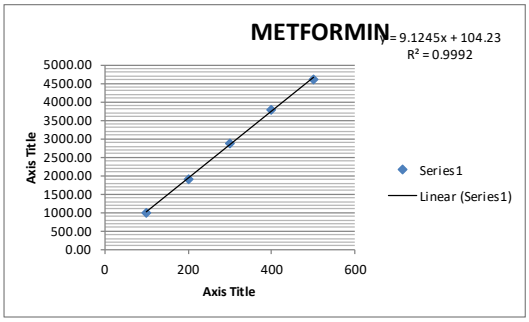

Figure 12: Representatie linearity of Metformin

b) Linearity result for Dapagliflozin

Linearity was established by the least-squares linear regression analysis of the calibration data. Calibration plots were linear over the concentration range of 2-10 µg/ml for Dapagliflozin Peak areas were plotted against the respective concentrations and linear regression analysis performed on the resulting curves. The linear curve of Dapagliflozin was shown in **Figure 9** respectively. The linear regression equation obtained was Y=45.06x+17.77 for Dapagliflozin with correlation coefficient 0.9993 respectively. The results of linearity are shown in Table 9

Table: 9 Linearity result for Dapagliflozin

Sr No	**Linearity Level**	**Concentration**	**Area**
1	I	2 µg/mL	107.93
2	II	4 µg/mL	196.48
3	III	6 µg/mL	295.15
4	IV	8 µg/mL	383.57
5	V	10 µg/mL	461.33
Correlation coefficient	R^2= 0.9993		

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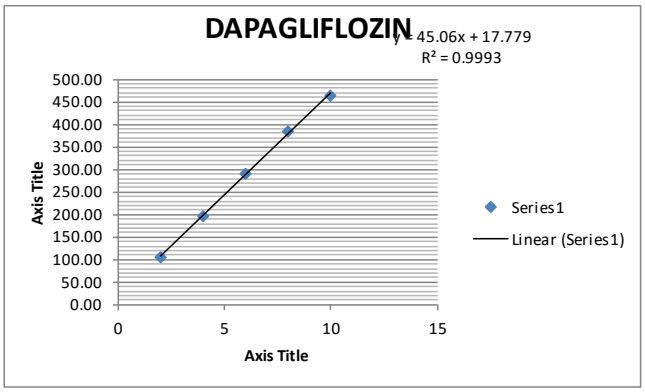

Figure 13: Representatie linearity of Dapagliflozin

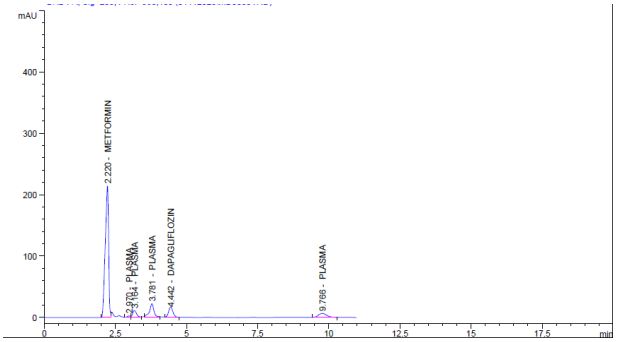

Figure 14: Representative Linearity Chromatogram of Met and Dapa with Plasma.

6. Robustness

Table no: 10 Robustness Study for Metformin & Dapagliflozin

Condition		Metformin		Dapagliflozin	
		SD	%RSD	SD	%RSD
Change in wavelength (210±1 nm)	232nm	1.55	0.04	0.56	0.13
	234nm	1.07	0.03	1.13	0.30
Change in flow rate (1.0±0.1 ml/min)	0.7ml	0.23	0.01	0.41	0.15
	0.9ml	2.90	0.09	0.21	0.06
Change in Mobile phase composition	49+51	4.74	0.12	3.17	0.20
	51+49	2.50	0.06	0.83	0.05

7. System Suitability parameters

Table no: 11 System Suitability parameters of drugs

Parameters	Dapagliflozin	Metformin
Peak area	107.25749	986.714
Throtical plate	4074	5601
Retention time	4.429	2.222
Tailing factor	0.90	1.84

Assay of tablet:

Assay of the marketed formulation having brand name (XIGDUO XR)was carried out by taking 20 tablets ,triturated it having average powder weight is 11.4gm.

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Weigh 20 Tablet and calculated the average weight, accurately weigh and transfer the sample equivalent to 285mg into 25 ml volumetric flask. Add about 10 ml MEOH of diluents and Sonicate to dissolve it completely and make volume up to the mark with diluent. Mix well and filter through 0.45 µm filter. Further pipette 0.4 ml of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluents. (400µg/ml+8µg/ml). The simple chromatogram of test Metformin and Dapagliflozin, The amounts of Metformin and Dapagliflozin per tablet were calculated by extrapolating the value of area from the calibration curve. Analysis procedure was repeated two times with tablet formulation. Tablet Assay for %Lable claim for %RSD Calculated, Result was shown in **(Table No. 12).**

Table no: 12 Analysis of marketed formulation.

Conc	**Area of Metformin**	**SD**	**%RSD**
400 µg/mL	3839.76	6.710	0.175
Conc	**Area of Dapagliflozin**	**SD**	**%RSD**
8.00µg/mL	381.92	2.299	0.602

Degradation studies

Table no: 13 Degradation studies of Drugs.

Sample name	**Area**	**Area Degraded**		**Sample name**	**Area**	**Area degraded**	
Metformin		**1 Hrs**	**After 2 Hrs**	**Dapagliflozin**		**1 Hrs**	**After 2 Hrs**
Acid	2896.92	2753.07	2620.22	Acid	290.49	274.66	253.786
Base	2896.92	2703.61	2533	Base	290.49	268.92	260.244
H_2O_2	2896.92	2716.15	2525.566	H_2O_2	290.49	271.5799	256.244
Neutral	2896.92	2895.12	2815.768	Neutral	290.49	289.2797	282.823

Percentage degradation

In these studies, we can interpret the acid, base, oxidation, degradation in the sample. In acid degradation when the drug interacts with acid it produces primary degradation in the desirable range. For acid analysis HCl or H2SO4 (0.1–1 M) is widely used. In basic degradation when the drug interacts with base it produces primary degradation in the desirable range. For base analysis, NaOH or KOH (0.1–1 M) is widely used. In oxidative degradation hydrogen peroxide is widely used for oxidation degradation. Drug structure will allow selecting concentration and condition of oxidizing agent.

Table no: 14 Actual Percentage degradation

Actual % Degradation of Metformin			**Actual % Degradation of Dapagliflozin**	
	1 Hr	**After 2 Hrs**	**1 Hr**	**After 2 Hrs**

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Acid	4.97	9.55	5.45	12.64
Base	6.67	12.56	7.72	10.41
H_2O_2	6.24	12.82	6.51	11.65
Neutral	0.06	2.80	0.42	2.64

Report on validation Parameters.

Table no : 15 Summary of validation parameter

Parameters	**Metformin**	**Dapagliflozin**
Linearity Range(**µgm/ml)**	100-500	2-10
Slope	9.12m	45.06m
Intercept	104.2c	17.77c
Regression	0.999	0.999
Accuracy(%Recovery)	100.5%	101.06%
Precession(%RSD)	0.056	0.40
Assay (%)	102.40	101.02
LOD	2.00	0.179
LOQ	6.06	0.545

Conclusions: It can be concluded that the developed Bioanalytical method is not reported for estimation of Dapagliflozin and Metformin combination spiked in human plasma. It also capable of quantifying Metformin and Dapagliflozin combination from spiked human plasma samples. The method meets the requirements of the ICH Guidelines and can be applied to Bioavailability or Bioequivalence studies. Based on the data presented in this report, it can be included that present method is validated for the estimation of Metformin in human plasma over a concentration range of 100-500 µg/ml & for Dapagliflozin 2 -10 µg/ml. The precision and accuracy are very much within the prescribed limits in this concentration range. Expected recoveries are observed in the present processing technique for 80%, 100%, and 120%.

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