



A Stability Indicating RP-HPLC Method Validation for Simultaneous Estimation of Metformin HCl, Dapagliflozin and Saxagliptin in Pharmaceutical Dosage Form

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Authors' contributions

This work was carried out in collaboration among all authors. Author DP designed the study, wrote the protocol, performed statistical analysis and wrote first draft of manuscript. Author US managed the final review and analysis of data. Authors JP, DP and PP managed the literature search. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Metformin HCl, Dapagliflozin and Saxagliptin is a new drug combination for the treatment of Diabetes Mellitus which is one of the oldest and lethal diseases of mankind. Aim of the research work was to develop and validate novel, rapid, sensitive, specific, robust stability indicating analytical method for simultaneous estimation of: Metformin HCl, Dapagliflozin and Saxagliptin in pharmaceutical dosage form as fixed dose formulation.

Study Design: Method development and validation was performed as recommended in ICH guideline "Validation of analytical procedures: Test and Methodology Q2 (R1)".

Methodology: Method develop with chromatographic parameters as C₁₈ column (250mm×4.6 mm, 5mm particle size), HPLC system with PDA detector and mobile phase contained a mixture of Phosphate Buffer pH 3.5 and Acetonitrile (80:20 v/v) + 1 ml triethylamine per 100 ml mobile phase.

The flow rate was set to 1 ml/min with responses measured at 265 nm, injection volume was 20 µl, and run time of 15 mins.

Results: The retention time of Metformin HCl, Dapagliflozin and Saxagliptin was 5.8 min, 6.8 mins and 8.4 min respectively with resolution of 3.5 between Metformin HCl and Dapagliflozin and 4.5 between Dapagliflozin and Saxagliptin. Linearity was established in the range of 250-1500 µg/ml for Metformin HCl, 1.25-7.5 µg/ml for Dapagliflozin and Saxagliptin with correlation coefficients more than 0.999. The percentage recoveries were between 98.39-101.66 for Metformin HCl, 99.01-101.77 for Dapagliflozin and 98.88-101.87 for Saxagliptin Validation parameters were evaluated according to the International Conference on Harmonization (ICH) Q2 R1 guidelines. The forced degradation studies were performed by using HCl, NaOH, H₂O₂, thermal and UV Radiation. The developed method was successfully applied for the quantification and hyphenated instrumental analysis.

Conclusion: Significance of developed method is that it can be utilize for routine or unknown sample analysis of assay of Metformin HCl, Dapagliflozin and Saxagliptin in pharmaceutical dosage form developed by various Pharmaceutical Industry.

Keywords: Metformin HCl; dapagliflozin; saxagliptin; RP-HPLC; stability; diabetes mellitus.

1. INTRODUCTION [1-11]

Diabetes Mellitus (DM) is an endocrinological disorder resulting from an irregularity in insulin secretion and insulin action or both. Absence or reduced insulin in turn leads to persistent abnormally high blood sugar and glucose intolerance. It is probably an oldest disease known to human. It is also referred as black-death from the 14th century. Diabetes mellitus mainly classified into two categories, Insulin dependent diabetes mellitus when the pancreas does not produce enough insulin to properly control blood sugar levels, in this condition the patient completely depends parental formulation of insulin another one Non-insulin dependent diabetes mellitus, the cells of the body become resistant to insulin which are treated with oral antidiabetic agents such as Sulfonylureas, Biguanides, Thiazolidinediones derivatives, carbohydrate analogue and DPP- 4 inhibitors. Fixed dose combination therapy (FDC) is called as a combination of two or more actives in a fixed ratio of doses. The International Diabetic Federation (IDA) and American Diabetic Federation (FDA) tend to suggest if the monotherapy fails along with lifestyle modification the patient should followed by combination therapy. Combination therapy based on the rationale of a multi targeted approach and it helps to achieve and maintain the desired therapeutic targets. The advantages of Fixed Dose Combination are easy of administration, convenience, synergistic effect, complementary mechanism of action, with low dose less side effects, economical, reduce the pill burden and thereby, improve adherence to treatment, improve tight glycemic control, decrease the

incidence/severity of Adverse Drug Reactions, delay the need for insulin therapy. Sodium-glucose cotransporter 2 (SGLT2) inhibitors such as Dapagliflozin, Biguanides such as Metformin HCl, Dipeptidyl Peptidase-4 (DPP-4) Inhibitor such as Saxagliptin exert antidiabetic effects via different mechanisms of action. SGLT2 inhibitors inhibit renal glucose reabsorption, resulting in increased urinary excretion of glucose and thereby reducing plasma glucose levels in an insulin independent manner. Biguanides does not have clear mechanism of action but it decreases glucose production and increases body's response to insuline. Dipeptidyl Peptidase-4 (DPP-4) Inhibitor Increased concentrations of the incretin hormones such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are released into the bloodstream from the small intestine in response to meals. These hormones cause insulin release from the pancreatic beta cells in a glucose-dependent manner but are inactivated by the DPP4 enzyme within minutes. GLP-1 also lowers glucagon secretion from pancreatic alpha cells, reducing hepatic glucose production. Because of the complementary mechanisms of action of SGLT2, Biguanides and DPP-4 therapy improves glycaemic control in patients with Type II Diabetes Mellitus.

FDC combination of Metformin HCl, Dapagliflozin and Saxagliptin is available with brand name of QTERNMET XR 1000 mg / 5 mg / 5 mg respectively.

Structure of Metformin HCl, Dapagliflozin and Saxagliptin are presented as Fig. 1, Fig. 2 and Fig. 3 respectively: [12-14].

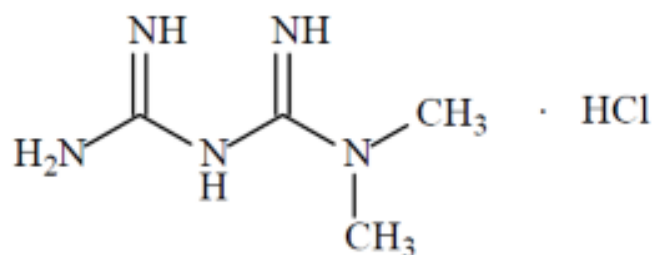


Fig. 1. Chemical Structure of Metformin HCl

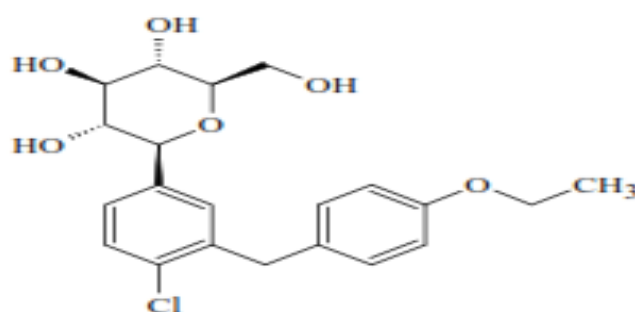


Fig. 2. Chemical Structure of Dapagliflozin

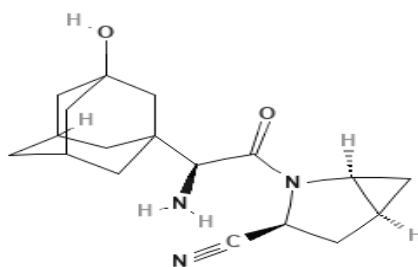


Fig. 3. Chemical Structure of Saxagliptin

After reviewing the literature, it can be said that individual analytical methods¹⁵⁻²⁴ are available for Metformin HCl, Dapagliflozin and Saxagliptin however, no RP-HPLC method is available for simultaneous estimation of Metformin HCl, Dapagliflozin and Saxagliptin in pharmaceutical dosage form as FDC. Developing Stability indicating RP-HPLC method for simultaneous estimation of Metformin HCl, Dapagliflozin and Saxagliptin in pharmaceutical dosage form as FDC is both requirement and challenge. In present work we have developed specific, stability indicating, linear, precise, accurate and robust analytical method for simultaneous

estimation of all three drugs in pharmaceutical dosage form as FDC.

2. MATERIALS AND METHODS

2.1 Chemical

The laboratory (working) standard of Metformin HCl, Dapagliflozin and Saxagliptin were received as gift sample from M/s Zydus Cadila. FDC product of Metformin HCl, Dapagliflozin and Saxagliptin was procured from market. Solvent like Water and Acetonitrile were HPLC grade and reagent such as Potassium dihydrogen

phosphate and triethylamine were analytical grade.

2.2 Analytical Method Development

2.2.1 Chromatographic conditions and instrument

Instrument : HPLC equipped with Photodiode-Array detector

Stationary Phase / Column : C18 Column, 250 mm X 4.6 mm, 5 μ : Phosphate Buffer pH 3.5: Acetonitrile (80:20) (1 ml Triethyl amine per 100 ml mobile phase)

Diluent : Acetonitrile

Flow Rate : 1 ml / min

Injection Volume : 20 μ l

Detection Wavelength : 265 nm

Column Temperature : Ambient

Run Time : 15 mins

Other Instruments : Analytical balance, sonicator, glassware

2.2.2 Preparation of solutions

2.2.2.1 Preparation of phosphate buffer pH 3.5

Dissolve 2.7 g of potassium dihydrogen phosphate in 900 ml of water and adjust pH to 3.5 with Phosphoric acid. Dilute to 1000 ml with water.

2.2.2.2 Preparation of mobile phase

Prepare a required volume of degassed mixture of Phosphate Buffer pH 3.5 and Acetonitrile in the ratio of 80:20 v/v. Add 1 ml of Triethylamine per 100 ml of mobile phase.

2.2.2.3 Preparation of standard stock solution for metformin HCl

Weigh 500 mg of Metformin HCl in 50 ml clean dry volumetric flask. To this add 50 mL of diluent and sonicate to dissolve. Dilute to volume with diluent and mix well.

2.2.2.4 Preparation of standard stock solution for dapagliflozin

Weigh 25 mg of Dapagliflozin in 50 ml clean dry volumetric flask. To this add 50 mL of diluent and sonicate to dissolve. Dilute to volume with diluent and mix well.

2.2.2.5 Preparation of standard stock solution for saxagliptin

Weigh 25 mg of Saxagliptin in 50 ml clean dry volumetric flask. To this add 50 mL of diluent and

sonicate to dissolve. Dilute to volume with diluent and mix well.

2.2.2.6 Standard solution

Transfer 10 mL of Standard Stock solution for Metformin HCl and 1 mL of Standard Stock Solution Dapagliflozin and Standard Stock solution Saxagliptin in 100 ml clean and dry volumetric flask, dilute to volume with diluent and mix well. Filter the solution with 0.45 μ PVDF / Nylon filter presaturated with diluent followed by discarding about 5 ml of initial solution. Final Concentration: Metformin HCl (1000 ppm), Dapagliflozin (5 ppm) & Saxagliptin (5 ppm).

2.2.2.7 Preparation of sample solution

Weigh about 20 intact tablets of Metformin HCl, Dapagliflozin and Saxagliptin 1000 mg / 5 mg / 5 mg Tablet FDC. Crush and transfer powder equivalent to 1000 mg Metformin HCl, 5 mg Dapagliflozin, 5 mg Saxagliptin in to dry and clean 200 ml volumetric flask. Add about 100 ml of diluent and sonicate at room temperature for about 15 mins with intermittent shaking at 5 min time interval. Further add about 50 ml diluent and sonicate at room temperature for 15 mins with intermittent shaking at about 5 min intervals. Dilute to volume with diluent and mix well. Equilibrate the solution to room temperature. Further dilute 5 ml of solution to 25 ml with diluent and mix well. Filter the solution through 0.45 μ PVDF / Nylon filter presaturated with diluent followed by discarding about 5 ml of initial solution. Final Concentration: Metformin HCl (1000 ppm), Dapagliflozin (5 ppm) & Saxagliptin (5 ppm).

2.3 Analytical Method Validation

The analytical method validation for was performed as per ICH guideline on "Validation analytical procedures: Text and Methodology Q2(R1)" [25]. Parameters such as System Suitability, specificity, forced degradation, linearity, precision, and accuracy, stability of solution and filter study were validated.

2.3.1 Criteria for system suitability

%RSD of area for replicate injections of standards, tailing factors, theoretical plates, retention time and resolution criteria were chosen as parameter for system suitability. Six replicate injection of standard were evaluated and chromatograms were recorded as part of system suitability study.

2.3.2 Specificity

Specificity was established by injecting blank, individual standard solution, combine standard solution and sample solution. Retention time and peak purity data were evaluated for individual analyte peak to confirm any interference.

2.3.3 Forced degradation study

To observe the effect of forced degradation and establish stability indicating nature of method sample preparation were kept under acid hydrolysis, base hydrolysis, oxidation, Photolytic exposure and Thermal exposure. After completing degradation study sample preparation was injected and studied for peak purity of individual peak of analyte in sample preparation.

Acid Hydrolysis: Sample was treated with 2mL 0.1N HCl at 85°C for 120 mins neutralize with 2mL 0.1N NaOH.

Base Hydrolysis: Sample was treated with 2mL 0.1N NaOH at 85°C for 120 mins neutralize with 2mL 0.1N HCl.

Oxidation: Sample was treated with 2 mL 30% H₂O₂(Hydrogen peroxide) at 85°C for 120 mins.

Thermal Degradation: Sample was treated at 105°C for 5 Days

Photo Degradation: Sample was treated for 1 day in UV chamber.

2.3.4 Precision

The precision was established based on three types of studies. i.e System Precision, Method Precision and Intermediate precision.

2.3.5 System precision

After establishing system suitability system precision was established by injecting six replicate injections of standard solution. The relative standard deviation for peak area should not be more than 2.0% for six replicate injections.

2.3.6 Method precision

After establishing system suitability Method precision was established by Established by preparing and injecting 6 samples at 100% concentration level. The % RSD of 6 samples at 100% concentration level should not be more than 2.0%.

2.3.7 Intermediate precision

After establishing system suitability Intermediate precision was established preparing and injecting 6 samples at 100% concentration level. The % RSD of 6 samples at 100% concentration level should not be more than 2.0%. The % RSD of result of method precision and intermediate precision (Total 12 samples at 100 % concentration level) should not be more than 2.0.

2.3.8 Linearity

Linearity is established on a minimum of 6 concentrations level across the range of 25-150% of standard solution concentration in duplicate. The standard solution at 6 different concentration levels in concentration range of 250-1500 µg/ml for Metformin HCl and 1.25-7.5 µg/ml for Dapagliflozin and Saxagliptin were prepared and injected in duplicate for each concentration level. The Linearity correlation coefficient obtains from the graph should not be less than 0.995.

2.3.9 Accuracy

The accuracy was established using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range from 25-150% (e.g., 3 concentrations/3 replicates). % recovery at all recovery level should be within the range of 98.0-102.0%. The %RSD at all recovery level should not be more than 2.0%.

2.3.10 Stability of solution

Stability of standard solution and sample solution were established by injecting same standard and sample solution at specific time intervals and comparing the peak area of the individual analyte over the time intervals.

2.3.11 Robustness

Robustness of the method was established by varying flow-rate, Buffer pH of mobile phase and Composition of mobile phase. System suitability was studied at each varied condition. System suitability criteria should be complied at each varied condition to prove robustness of method.

3. RESULTS AND DISCUSSION

3.1 Method Development

UV spectra of Metformin HCl, Dapagliflozin and Saxagliptin show good absorption at common wavelength of 265 nm. Therefore 265 nm was selected as detection wavelength. The retention

time of Metformin HCl, Dapagliflozin and Saxagliptin were found 5.8, 6.8 and 8.4 minutes respectively at flow rate of 1.0 ml/minutes in isocratic mode. The well resolved peak with resolution factor was found to be around 3.5 between Metformin HCl and Dapagliflozin peaks and 4.5 between Dapagliflozin and Saxagliptin peaks. (Fig. 4 to 10).

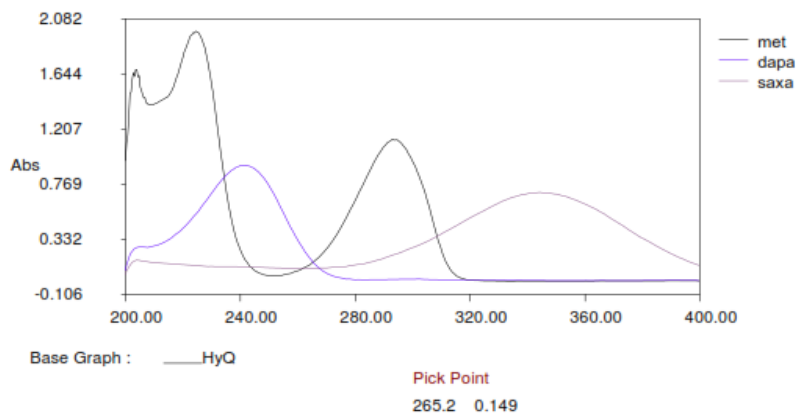


Fig. 4. Overlay US Spectra of Metformin HCl, Dapagliflozin and Saxagliptin

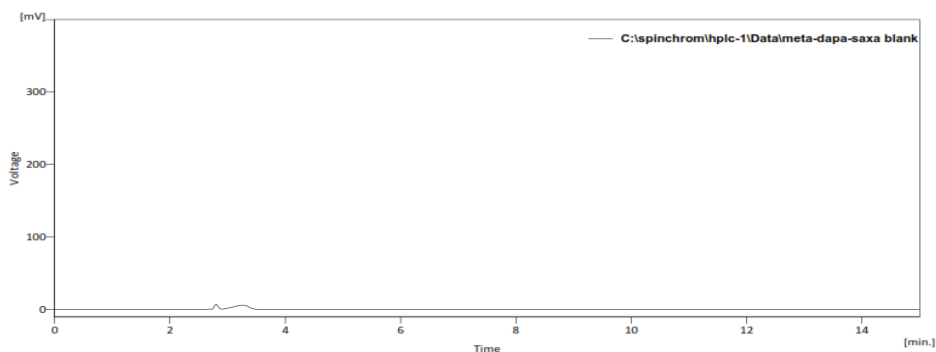


Fig. 5. Chromatogram of Blank Preparation

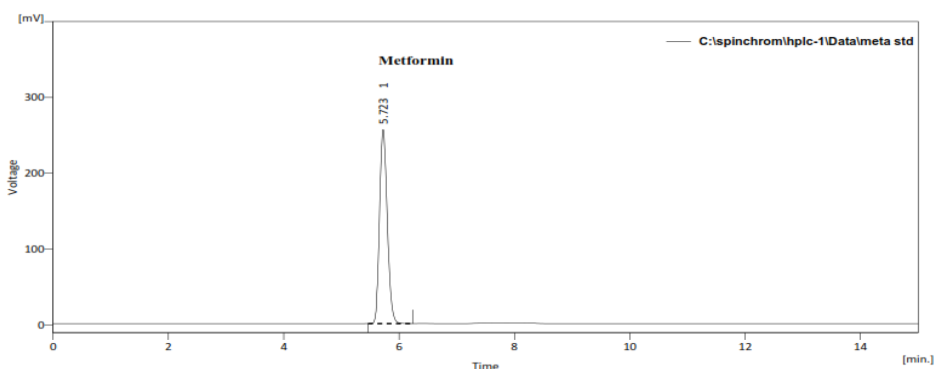


Fig. 6. Chromatogram of Metformin HCl Individual Standard Solution:

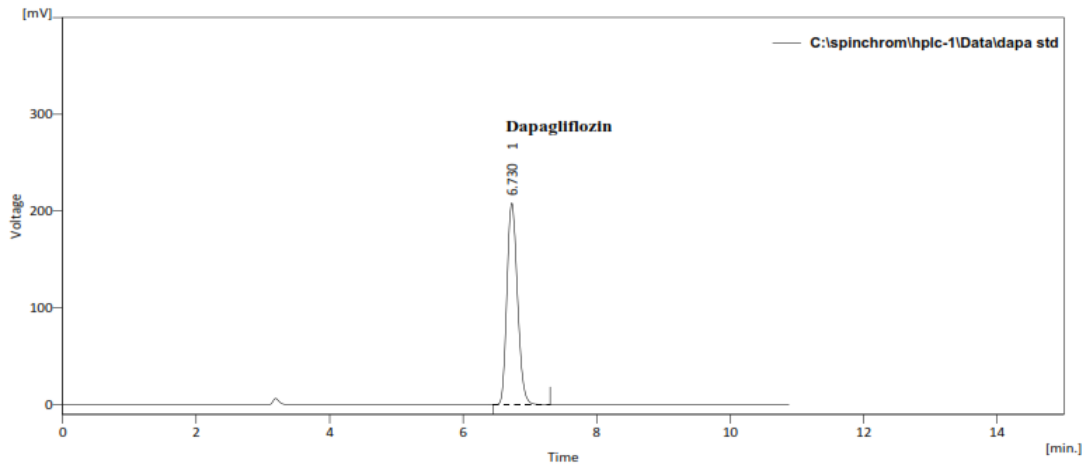


Fig. 7. Chromatogram of Dapagliflozin Individual Standard Solution:

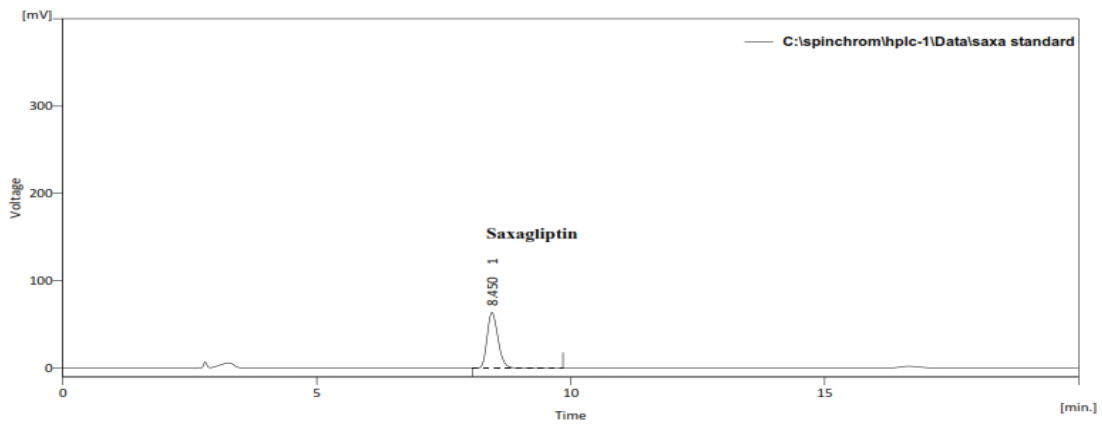


Fig. 8. Chromatogram of Saxagliptin Individual Standard Solution:

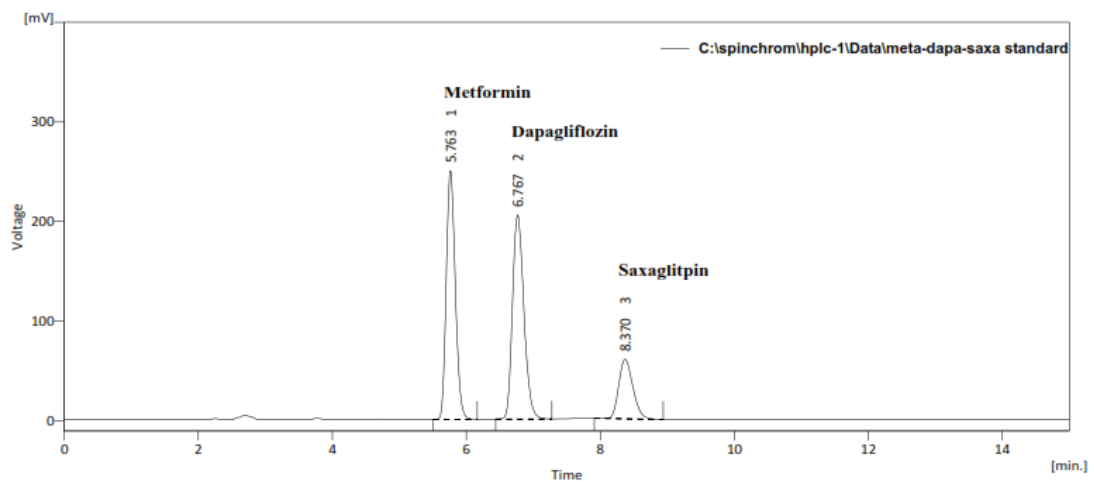


Fig. 9. Chromatogram of Combined Standard Solution:

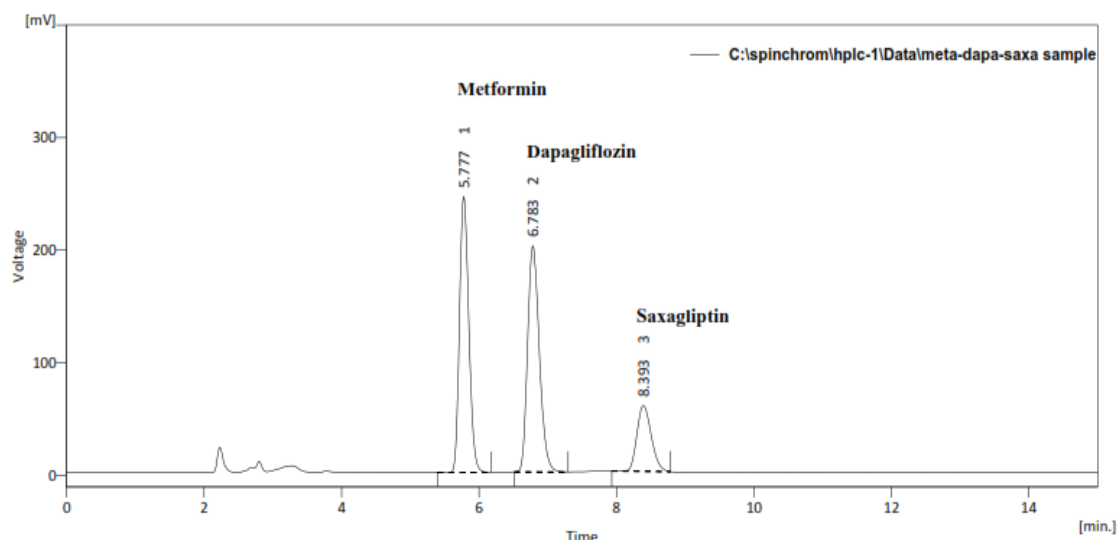


Fig. 10. Chromatogram of Sample Solution:

Table 1. Summary of System Suitability results

Peak	Retention Time	Theoretical Plates	Tailing Factor	%RSD	Resolution
Metformin HCl	5.76	1.3	8179	0.51	-
Dapagliflozin	6.77	1.3	7280	0.29	3.5
Saxagliptin	8.37	1.4	7337	1.25	4.5
Acceptance Criteria	-	NLT 5000	Between 0.8-2.0	NMT 2.0%	NLT 2.5

3.1.1 System suitability

For the system suitability to pass few criteria were set according to which the theoretical plate for Metformin HCl, Dapagliflozin and Saxagliptin peak should not be less than 5000, a tailing factor should be between 0.8 to 2.0, % RSD of six replicate injection should not be more than 2.0 and resolution factor between two adjacent peaks should be more than 2.5. Results of System Suitability observed for proposed method were summarized below in Table 1.

Based on above summarized results it is evident that system suitability meets predefined acceptance criteria.

3.1.2 Specificity

Retention times of individual peak of drugs in standard solution were corresponding to that of sample solution. Peak purity angle was less than peak purity threshold for individual peak of drugs

in standard solution and sample solution. Result evident that method is specific for its intended use.

3.1.3 Forced degradation study

During forced degradation study it was observed that Metformin HCl, Dapagliflozin and Saxagliptin were susceptible to acid, base, oxidation, Thermal and photo degradation. Summary of forced degradation study of Metformin HCl, Dapagliflozin and Saxagliptin is summarized in Table 2. Purity Angle is less than Purity Threshold for individual peak of Metformin HCl, Dapagliflozin and Saxagliptin in all degradation samples. Based on this it is concluded that there is no interference with individual drug peak due to any other peak in all degradation samples. Hence, it is evident that analytical method is stability indicating in nature. Chromatograms of forced degradation study are presented in Fig. 11 to 15.

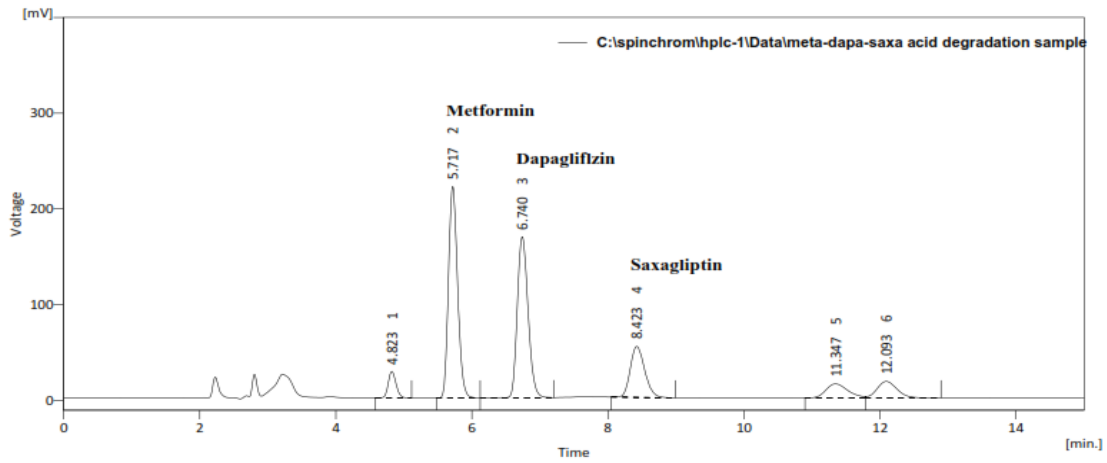


Fig. 11. Chromatogram of Acid Degradation Sample

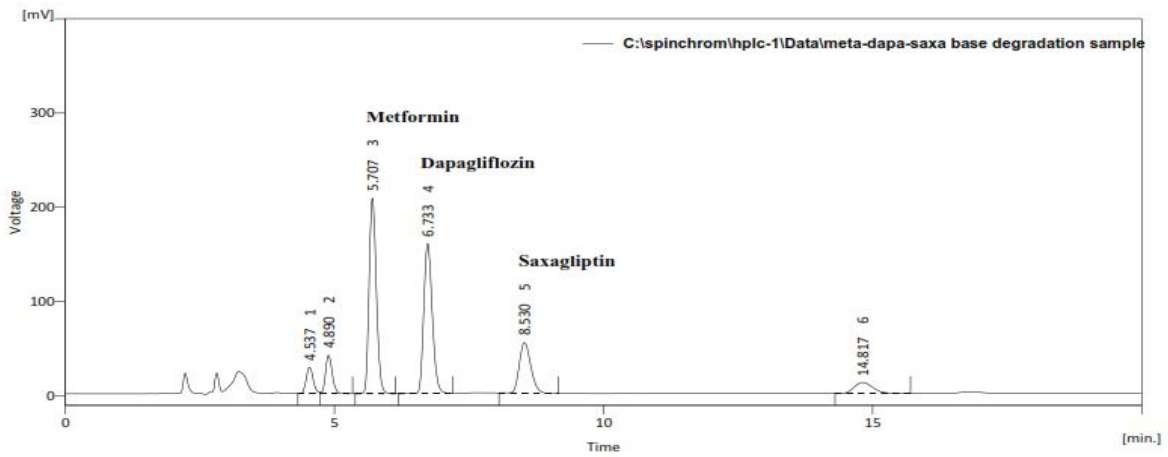


Fig.12. Chromatogram of Base Degradation Sample

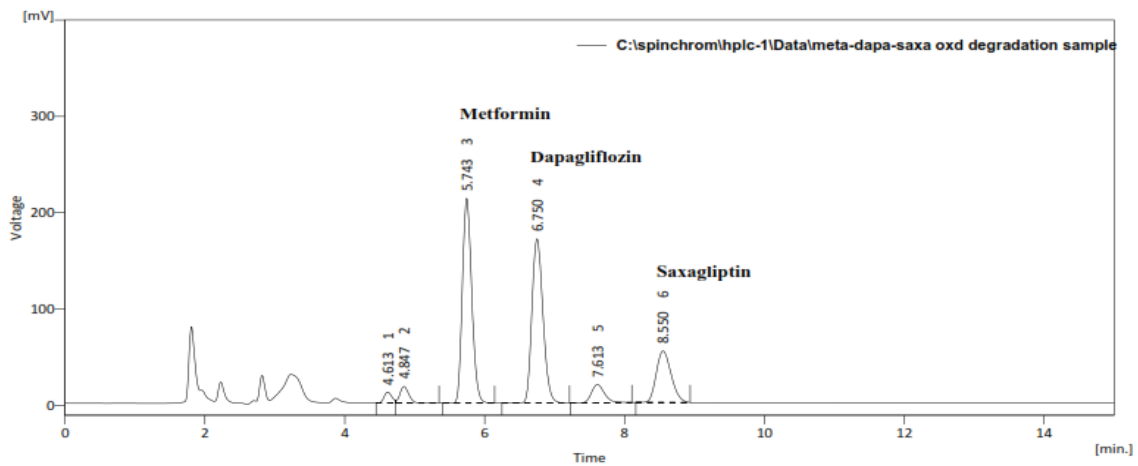


Fig. 13. Chromatogram of Oxidation Degradation Sample

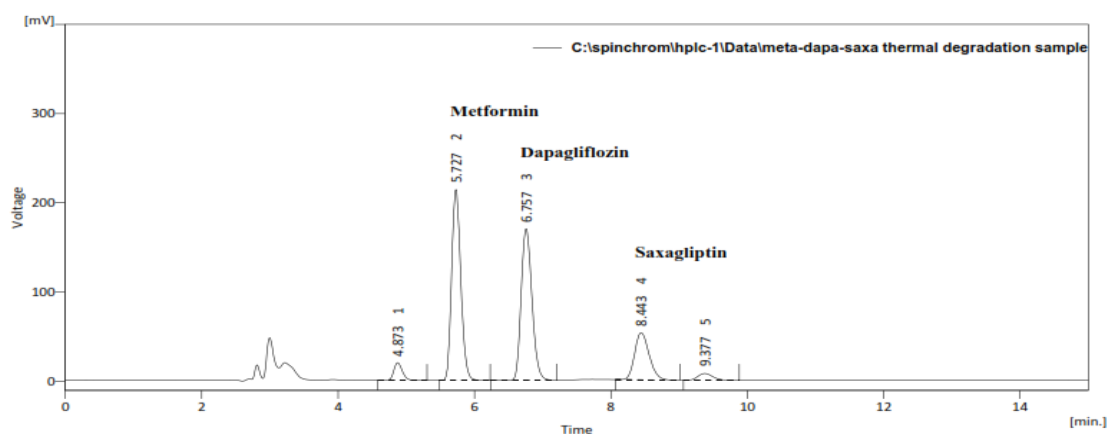


Fig. 14. Chromatogram of Thermal Degradation Sample

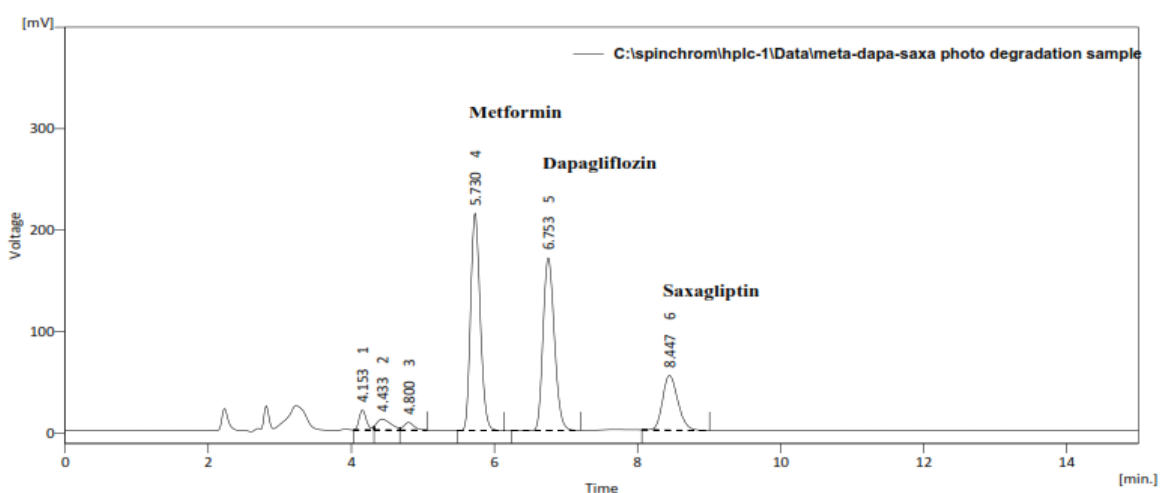


Fig. 15. Chromatogram of Photo Degradation Sample

Table 2. Summary of Force degradation study of Metformin HCl, Dapagliflozin and Saxagliptin

Degradation Condition	Metformin HCl		Dapagliflozin		Saxagliptin	
	% Assay	% Degd.	% Assay	% Degd.	% Assay	% Degd.
Undegraded Sample	100.30	-	101.30	-	99.63	-
Acid degradation	86.41	13.89	82.24	19.06	83.47	16.16
Base degradation	81.10	19.20	77.65	23.65	85.26	14.37
Oxidation degradation	83.57	16.73	83.59	17.71	83.88	15.75
Thermal Degradation	83.79	16.51	82.99	18.31	82.97	16.66
Photo Degradation	83.93	16.37	83.11	18.19	84.39	15.24

3.1.4 Precision

3.1.4.1 System Precision

System precision was established by injecting six replicate injections of standard preparation. %RSD of six replicate injection of standard

preparation was below 0.64% for Metformin HCl, Dapagliflozin and Saxagliptin.

3.1.4.2 Method Precision

Method precision was established by injecting 6 samples at 100% concentration level and

comparison of closeness between 6 results. % RSD of 6 samples at 100% concentration level was 0.44, 0.35 and 0.23 for Metformin HCl, Dapagliflozin and Saxagliptin respectively.

Summary of result for Method Precision is summarized in Table-3.

3.1.4.3 Intermediate Precision

Intermediate precision was established by injecting 6 samples at 100% concentration level and comparison of closeness between 6 results. % RSD of 6 samples at 100% concentration level was 0.78, 0.31 and 0.90 for Metformin HCl, Dapagliflozin and Saxagliptin respectively. The % RSD of result of method precision and intermediate precision (Total 12 samples at 100 % concentration level) was below 0.60, 0.64 and 1.1 Metformin HCl, Dapagliflozin and Saxagliptin respectively.

Summary of result for Intermediate Precision is summarized in Table-3.

3.1.5 Assay of Market Formulation

Market FDC of Metformin HCl, Dapagliflozin and Saxagliptin was analyzed and % Assay found 100.30% and 101.30 and 99.63% for Metformin HCl, Dapagliflozin and Saxagliptin Respectively.

3.1.6 Linearity

The calibration curve for Metformin HCl, Dapagliflozin and Saxagliptin was found linear over the concentration range of 250-1500 µg/ml for Metformin HCl and 1.25-7.5 µg/ml for Dapagliflozin and Saxagliptin. The regression coefficient value was found more 0.9997, 0.9997 and 0.9995 for Metformin HCl, Dapagliflozin and Saxagliptin respectively. Calibration curve for Metformin HCl, Dapagliflozin and Saxagliptin are provided in Fig. 16 Fig. 17 and Fig. 18 respectively. Statistical Parameter observed during Linearity study is summarized in Table 4.

Table 3. Summary of Method Precision and Intermediate Precision Study

Sample ID	Precision			Intermediate Precision		
	Metformin HCl	Dapagliflozin	Saxagliptin	Metformin HCl	Dapagliflozin	Saxagliptin
1	1010.57	5.01	5.00	1013.92	4.98	5.03
2	1017.59	5.04	5.02	1003.82	4.96	4.98
3	1009.46	5.01	5.00	998.56	4.99	4.94
4	1006.78	5.00	5.03	998.12	5.01	5.06
5	1004.57	5.03	5.06	1004.59	5.08	5.15
6	1013.28	5.04	4.96	1011.16	4.99	5.06
Mean	1012.54	5.02	5.01	1005.43	4.98	4.98
SD	4.41	0.02	0.01	7.81	0.02	0.05
%RSD	0.44	0.35	0.23	0.78	0.31	0.90
Overall %RSD	-	-	-	0.60	0.64	1.11

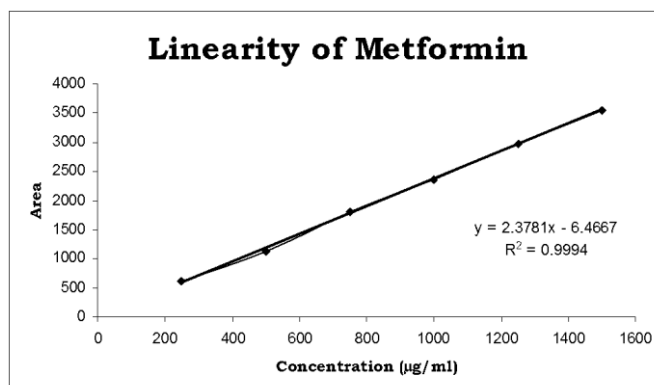


Fig. 16. Linearity Calibration curve for Metformin HCl

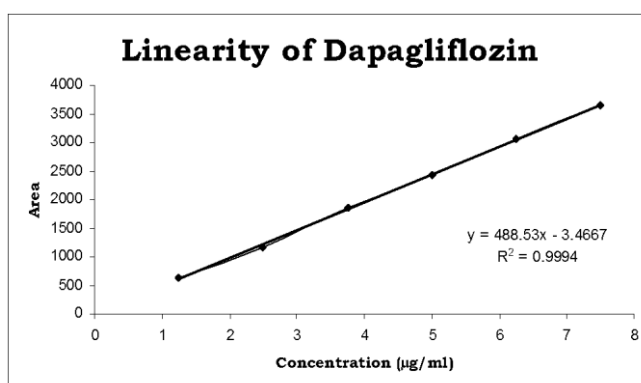


Fig. 17. Linearity Calibration curve for Dapagliflozin

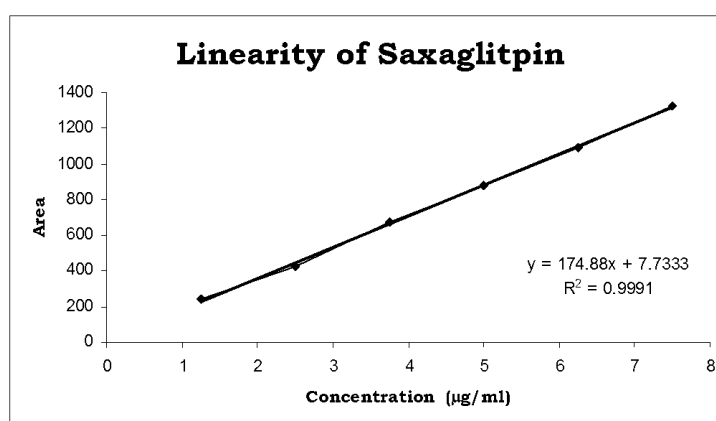


Fig. 18. Linearity Calibration curve for Saxagliptin

Table 4. Statistical Parameter observed during Linearity study

Regression analysis	Metformin HCl	Dapagliflozin	Saxagliptin
Linearity range	250-1500 µg/ml	1.25-7.5 µg/ml	1.25-7.5 µg/ml
Regression equation	$y = 2.3781x - 6.4667$	$y = 488.53x - 3.4667$	$y = 174.88x + 7.7333$
Correlation co-efficient	0.9997	0.9997	0.9995
Intercept	2.3781	488.53	174.88
Slope	-6.4667	-3.4667	7.7333

Table 5. Summary of result of method validation Parameters

Parameter	Metformin HCl	Dapagliflozin	Saxagliptin
Specificity	No Interference	No Interference	No Interference
Range	250-1500 µg/ml	1.25-7.5 µg/ml	1.25-7.5 µg/ml
System Precision (%RSD)	0.48	0.64	0.50
Method Precision (%RSD)	0.44	0.35	0.23
Intermediate precision (%RSD)	0.78	0.31	0.90
Overall Precision (%RSD)	0.60	0.64	1.11
Linearity (correlation co-efficient)	0.9997	0.9997	0.9995
Accuracy (% Recovery)	98.39-101.66%	99.01-101.77%	98.88-101.87%
Robustness (%RSD)	Meets System Suitability Acceptance Criteria		
% Assay	100.30%	101.30%	99.63%

3.1.7 Accuracy

The accuracy of the assay method was established three levels i.e. 25%, 100% and 150% of sample concentration. % Recovery observed between 98.39-101.66%, 99.01-101.77 and 98.88-101.87% for Metformin HCl, Dapagliflozin and Saxagliptin respectively. % RSD observed for accuracy was well below 2.0%.

3.1.8 Stability of Solution

Stability of standard and sample preparation was established by examining the peak area at 0 Hrs, 8 Hrs, 24 Hrs and 48 Hrs time intervals and % change in area of peak was monitored with time. No significant change in peak area was observed in peak area till 48 Hrs for standard and sample preparation.

3.1.9 Robustness

The robustness of method was established by varying flow-rate, pH of Mobile Phase buffer and ratio of Mobile Phase. System suitability was performed by varying the method parameters. System suitability criteria was in compliance to predefine limit of theoretical plate should not be less than 5000, a tailing factor should be between 0.8 to 2.0, % RSD of six replicate injection should not be more than 2.0 and resolution factor between two peak adjacent peaks should be more than 2.5.

Summary of results for all method validation parameters are provided in Table 5. Based on result of method validation it is established that proposed RP-HPLC is simple, specific, stability-indicating, Linear, Precise, Accurate and robust.

4. CONCLUSION

A novel, rapid, sensitive, specific, robust stability indicating RP-HPLC method was developed and validated for Metformin HCl, Dapagliflozin and Saxagliptin fixed dose formulation. All method validation parameter lie within its acceptance criteria as per ICH Q2 (R1) Guideline. So, it can conclude that method is selective, linear, accurate and precise. There is no co-elution of any degradation products with main peak and results obtained were found within the acceptance criteria. Hence, the method can be termed as selective. Therefore, the proposed RP-HPLC assay method can be applied for the estimation of Metformin HCl, Dapagliflozin and

Saxagliptin in pharmaceutical dosage form in the presence of degradation products.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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