Research Article

Method Development and Validation of A Stability-Indicating Reversed-Phase Liquid Chromatographic Method for the Simultaneous Estimation of Metformin and Dapagliflozin in Presence of their Degradation Products

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Received: 26-04-2018; **Revised:** 03-05-2019; **Accepted:** 21-05-2019.

ABSTRACT

A reversed-phase high-performance liquid chromatographic method was developed and validated for the simultaneous estimation of Metformin and Dapagliflozin in the presence of their degradation products. Analytes were separated on a Hypersil C18, 250x4.6 mm, 5µm column using an isocratic elution mode having mobile phase composition of 50 mM potassium dihydrogen phosphate buffer (pH 3.0): methanol (40:60 %v/v). The analytes were detected at a wavelength of 255 nm. A 20µL fixed-loop injector was used for the injection of the samples with a flow rate of 1.0 mL min−1. The optimized method was validated as per ICH Q2 guidelines. The retention times of Metformin and Dapagliflozin were 3.78 min and 5.74 min, respectively. The linearity was 5-20 µg/ml for each of Metformin and Dapagliflozin, respectively. The correlation coefficient for calibration curves of both Metformin and Dapagliflozin were >0.99. Accuracy was 98-102%. Inter and Intra-day precision were calculated <2 %RSD. Limit of detection (LOD) and limit of quantitation (LOQ) were within the limits of ICH-Q2 guidelines. The method was robust with % RSD values <2% with the deliberate changes in the composition of mobile phase, changes in the pH or change in the flow rate. Significant degradation observed in the presence of acidic, basic, neutral, oxidative and photolytic stress conditions. The proposed RP-HPLC method is simple, precise, accurate, robust and reproducible and was able to successfully separate and quantify Metformin and Dapagliflozin in the presence of their degradation product, which implies the stability indicating nature and specificity of the method.

Keywords: Metformin, Dapagliflozin, stress testing, degradation products, stability-indicating method, HPLC.

INTRODUCTION

Type 2 Diabetes mellitus (T2DM) is the most prevalent metabolic disease worldwide. Inadequate management and control of hyperglycemia in patients with T2DM may lead to the risk of developing complications over the long term due to chronic and progressive nature of the disease arising from pathophysiology of beta-cell dysfunction, insulin resistance and increased hepatic glucose output. A combined formulation consisting of both Metformin and Dapagliflozin in a single tablet would potentially offer increased patient convenience and subsequent potential for increased therapeutic compliance. Xigudo XR (AstraZeneca, Inc.) is an immediate release (IR) fixed-dose combination (FDC) tablet of Dapagliflozin (5 or 10 mg) and Metformin (500, 850, or 1000 mg) that has received regulatory approval in the European Union and United states for the treatment of adults with T2DM to improve glycemic control in patients not adequately controlled on their maximally tolerated doses of Metformin alone or combined with other glucose-lowering medicinal products including insulin, and patients already treated with combination of Dapagliflozin and Metformin as separate immediate release tablets.[3]

Various ultraviolet spectroscopic and high performance liquid chromatographic assay methods were reported for the estimation of Metformin and Dapagliflozin individually and in combination with other drugs. The report presents the development and validation of stability indicating reversed-phase liquid chromatographic method for simultaneous estimation of Metformin and Dapagliflozin and all possible degradation products in combined dosage forms in accordance with the ICH guidelines.[2] Several chromatographic and spectrophotometric methods for the simultaneous estimation of Metformin and Dapagliflozin are reported.[4-13] This is the report of simple, precise, accurate, sensitive and reproducible stability indicating a method for the simultaneous estimation of Metformin and Dapagliflozin.

Advantages of development of stability indicating method development for the simultaneous estimation in the mixture of drugs are the identification of new impurities in addition to those studied for stability assays of Metformin and Dapagliflozin alone. The methods can be used to understand mutual induction or inhibition of rates of degradation and to analyze common impurities of both drugs in combined dosage forms.

MATERIALS AND METHODS

Instrumentation

Analytes were scanned between 200-400 nm using UV-visible spectrophotometer (Shimadzu, model UV-1700). Experiments were carried out using Shimadzu prominence Modular HPLC system with LC 20AT solvent delivery unit,

CBM 20A system controller, SIL 20A auto-sampler, CTO 20A column oven and SPD 20 A UV Detector. Data was recorded and evaluated using Spinchrom software as the data integrator. 20µL fixed-loop injector was used for the injection of the samples with the flow rate of 1.0 mL min−1. The pH of the solutions was measured with the pH meter (Mettler Toledo, S20K). Refluxing of the drugs in specific degradation conditions were carried out using a Rotavapor (R-300, Buchi). A Shimadzu ATX-124 analytical balance was used for weighing.

Reagents and Chemicals

The Metformin and Dapagliflozin reference materials were purchased from Mesochem Technology, Inc., Beijing, China. Methanol and Water were used of HPLC grade and purchased from Fisher Scientific, India. Potassium dihydrogen phosphate buffer was purchased from Sigma-Aldrich Company, India.

Selection of wavelength

Standard solution of Metformin (10 µg/mL) and Dapagliflozin (10 µg/mL) were scanned between 200-400 nm using a UV-visible spectrophotometer. Wavelength was selected from the overlay spectra of above solutions.

Chromatographic separation

Analytes were separated on Hypersil C18, 250x4.6 mm, 5µm column using an isocratic elution mode having mobile phase composition of 50 mM potassium dihydrogen phosphate buffer (pH 3.0): methanol (40:60 %v/v). The detection was carried out at the wavelength of 255 nm. Peak area, peak height, retention time and resolution were recorded using Spinchrom software. 20µL fixed-loop injector was used for the injection of the samples with the flow rate of 1.0 mL min−1.

Preparation of standard solutions

10 mg of Metformin and 10 mg of Dapagliflozin were separately weighed and transferred to previously labeled 100 mL volumetric flasks and volume was made up to the mark with methanol to obtain 100 µg/mL of Metformin standard stock solution and 100 µg/mL of Dapagliflozin standard stock solution. 1 mL from the Metformin stock solution and 1mL from Dapagliflozin stock solution were transferred into labeled 10 mL volumetric flasks and volume was made up to the mark by mobile phase to obtain a standard solution of mixtures of 10 µg/mL for each Metformin and Dapagliflozin, respectively.

Method Validation

System suitability test

System suitability test is an integral part of the chromatographic method. These tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as a whole. System suitability testing provides assurance that the method will provide accurate and precise data for its intended use.

Linearity

The linearity was assessed by analysis of combined standard solution in a range of 5- 20 µg/ml for each of the Metformin and Dapagliflozin, respectively.

Precision

Results were expressed as percentage relative standard deviation (%RSD) or coefficient of variance.

Repeatability

A standard solution containing 10 µg/ml of each of the Metformin and Dapagliflozin, respectively, was injected six times. The peak areas were measured and % RSD was calculated to determine the repeatability of the method.

Intra- day and inter-day precision

A standard solution containing 5, 10 and 15 µg/ml of each of the Metformin and Dapagliflozin were analyzed three times on the same day for the determination of intra-day precision and on three different days for the determination of inter-day precision and % R.S.D was calculated.

Accuracy

Accuracy was calculated at three different levels in terms of % recovery by spiking known amount of standard solution (80%, 100% and 120%) to the solution of a synthetic laboratory mixture of Metformin and Dapagliflozin.

Specificity and selectivity

The specificity of the method was established through the study of resolution factors of the drug peak from the nearest resolving peak and also among all other peaks.

Limit of detection and Limit of quantitation (LOD and LOQ)

The LOD and LOQ were estimated at signal-to-noise ratios of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations.

Robustness

Robustness of the method was investigated by varying the chromatographic conditions, such as, changing the flow rate by ± 10% i.e. 0.8 ml/min and 1.2 ml/min; changing the ratio of mobile phase was with ±2 i.e. buffer: methanol (38:62) and buffer: methanol (42:58); and changing the pH of the buffer in the mobile phase with ± 0.2% i.e. 2.8 and 3.2. Robustness of the developed method was indicated by the overall % RSD between the data, at each variable condition.

Analysis of synthetic laboratory mixture

Synthetic laboratory mixture of with 10 mg of Metformin and 10 mg of Dapagliflozin were weighed individually and spiked with 1 mg Hydroxy Propyl Cellulose (E463) and 1 mg Micro Crystalline Cellulose (E460 (i)) as tablet excipients into a 100 ml volumetric flask. The analytes were extracted with 5 ml methanol by sonication in the ultra-sonicator bath and then the volume was made up to the mark with mobile phase. The solution was filtered through Whatman filter paper no. 42. One mL from this solution was transferred to 25 ml volumetric flask and volume was made up to the mark with mobile phase to obtain the concentration of 10 µg/ml of Metformin and Dapagliflozin each respectively. Samples were analyzed using the developed assay. The areas of resulting peaks were measured at 255 nm.

Stress degradation studies

Acid hydrolysis

Forced degradation in acidic condition was performed by adding 1 ml of standard solution of mixtures of Metformin (1 mg/mL) and Dapagliflozin (1 mg/mL) to 10 ml each of methanol and 0.1 M hydrochloric acid and refluxing the mixture at 70°C for 4 hours (n=3). The solution was then allowed to reach at room temperature, neutralized to pH 7 by the addition of 0.1 M sodium hydroxide, and diluted to 100 ml with the mobile phase so as to get a final concentration of 10 µg/ml for each of Metformin and Dapagliflozin respectively. Synthetic laboratory mixture equivalent to 10 mg of Metformin and 10 mg Dapagliflozin was also treated with described acidic conditions.

Alkaline hydrolysis

Alkali-induced, forced degradation was performed by adding 1 ml of a standard solution of a mixture of Metformin (1 mg/mL) and Dapagliflozin (1 mg/mL) to 10 ml each of methanol and 0.1 M sodium hydroxide and refluxing the mixture at 70°C for 2 hours. The solution was then allowed to reach at room temperature, neutralized to pH 7 by the addition of 0.1 M hydrochloric acid, and diluted to 100 ml with the mobile phase to get a final concentration of 10 µg/ml for each of Metformin and Dapagliflozin respectively. Synthetic laboratory mixture equivalent to 10 mg of Metformin and 10 mg Dapagliflozin was also treated with described alkaline conditions.

Oxidative degradation

To evaluate the effect of oxidizing conditions, 1 ml of the standard solution of a mixture of Metformin (1 mg/mL) and Dapagliflozin (1 mg/mL) was added to 2 ml of 3% hydrogen peroxide solution and the mixture was refluxed at 70°C for 2 hours. The solution was then allowed to reach room temperature and diluted to 100 ml with the mobile phase to get a final concentration of 10 µg/ml for each of Metformin and Dapagliflozin respectively. Synthetic laboratory mixture equivalent to 10 mg of Metformin and 10 mg Dapagliflozin was also treated with described oxidative degradation conditions.

Thermal degradation

To evaluate the effect of temperature, 1 ml of a standard solution of a mixture of Metformin (1 mg/mL) and Dapagliflozin (1 mg/mL) was stored at 105°C in a hot air oven for 1.5 hours. The solution was then allowed to reach room temperature and diluted to 100 ml with the mobile phase to get a final concentration of 10 µg/ml for each of Metformin and Dapagliflozin respectively. Synthetic laboratory mixture equivalent to 10 mg of Metformin and 10 mg Dapagliflozin was also treated with described thermal degradation condition.

Photolytic degradation

To study the effect of UV light, a mixture of Metformin and Dapagliflozin, 25 mg each, was exposed to short and long wavelength UV light (254 nm and 366 nm, respectively) for 24 hours, and then dissolved in 10 ml of methanol. The volume was made up by the mobile phase in a 50 ml volumetric flask and then 1 ml of stock solution was further diluted with the mobile phase to give a solution of final concentration equivalent to 10 µg/ml for each of Metformin and Dapagliflozin. Synthetic laboratory mixture equivalent to 10 mg of Metformin and 10 mg Dapagliflozin was also treated with described photolytic degradation conditions.

Twenty microliters of the resulting solutions were injected into the HPLC system and the chromatograms were recorded.

RESULTS AND DISCUSSION

Method development

As Metformin and Dapagliflozin both showed absorbance response at a wavelength of 255 nm, it was selected as a wavelength of detection.

The analytes were separated on Hypersil C18, 250 x 4.6 mm, 5µm column using an isocratic elution mode having mobile phase composition of 50 mM potassium dihydrogen phosphate buffer (pH 3.0): methanol (40: 60 %v/v) and detected at 255 nm. 20µL fixed-loop injector was used for the injection of the samples with the flow rate of 1.0 mL min−1. Retention times were 3.78 min and 5.74 min for Metformin and Dapagliflozin respectively, as shown in Figure 1.

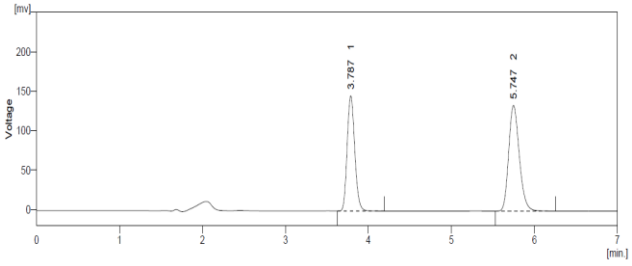

Figure 1: Chromatogram of Metformin[1] and Dapagliflozin[2] in 50 mM potassium di-hydrogen phosphate buffer (pH 3.0): methanol (40:60 v/v) with flow rate-1.0 ml/min

Method validation

The method was validated as per ICH guidelines[2] with respect to parameters defining linearity, precision, accuracy, specificity, and robustness.

The number of theoretical plates, peak tailing and resolution factor were determined to define system suitability parameters for Metformin and Dapagliflozin. The results for system suitability data are listed in Table 1. Linearity and range were assessed by analysis of combined standard solution in the range of 5-20 µg/ml for Metformin and Dapagliflozin, each respectively. The data for regression analysis is listed in Table 2.

Table 1: System suitability parameters for Metformin and Dapagliflozin

System Suitability Parameters	Metformin	Dapagliflozin
Theoretical plates per column (N)	7439	9334
Symmetry factor/ Tailing factor	1.240	1.364
Resolution	9.479	

Table 2: Results from regression analysis for Metformin and Dapagliflozin

Description	Metformin	Dapagliflozin
Linearity and range	5-20 µg/ml	5-20 µg/ml
Regression co-efficient	0.999	0.999
Slope (m)	92.03	118.2
Intercept (c)	8.28	8.29

A standard solution containing 10 µg/ml for each of Metformin and Dapagliflozin respectively was injected six times and areas of peaks were measured to determine the repeatability of the method. % R.S.D. value for the determination of repeatability is represented in Table 3. A standard solution containing 5,10 and 15 µg/ml for each Metformin and Dapagliflozin were analyzed three times on the same day for the determination of intra-day precision and on three different days for the determination of inter-day precision. % R.S.D values for intra-day and inter-day precision are represented in Table 4. The accuracy of the method was confirmed by recovery study from the synthetic mixture of marketed formulation at three levels of standard addition. The results are shown in Table 5.

Table 3: Repeatability data for Metformin and Dapagliflozin

Metformin				Dapagliflozin			
Conc. (µg/ml)	Peak Area	Mean ± S.D (n=6)	% R.S.D	Conc. (µg/ml)	Peak Area	Mean ± S.D (n=6)	% R.S.D
10	924.40	923.44±5.27	0.57	10	1186.16	1183.56±6.44	0.54
	926.25				1188.53		
	929.00				1192.10		
	927.12				1181.26		
	917.87				1177.83		
	916.02				1175.47		

Table 4: Intra-day and Inter-day precision for Metformin and Dapagliflozin

Metformin			Dapagliflozin		
Conc. (µg/ml)	Mean ± S.D (n=6)	% R.S.D	Conc. (µg/ml)	Mean ± S.D (n=6)	% R.S.D
Intra-day precision					
5	467.69 ± 1.50	0.32	5	598.66 ± 4.55	0.76
10	924.37± 1.86	0.20	10	1184.54±4.98	0.42
15	1382.66± 6.62	0.47	15	1772.60±9.25	0.52
Inter-day precision					
5	468.60 ± 3.35	0.71	5	600.20 ± 4.81	0.803
10	915.08± 15.83	1.73	10	1180.22±10.67	0.904
15	1383.00±11.60	0.80	15	1773.05±17.31	0.976

Table 5: Accuracy in terms of % recovery for Metformin and Dapagliflozin

Conc. Level (%)	Sample amount (µg/ml)	Amount of Standard Added (µg/ml)	Metformin			Dapagliflozin		
			Amount Recovered (µg/ml)	% Recovery	% Mean Recovery ± S.D	Amount Recovered (µg/ml)	% Recovery	% Mean Recovery ± S.D
80 %	5	4	4.04	101.0	100.3 ± 0.58	4.03	100.9	100.1 ± 0.85
	5	4	3.99	99.9		3.97	99.2	
	5	4	4.00	100.1		4.00	100.1	
100 %	5	5	5.02	100.5	100.7 ± 0.62	5.02	100.4	100.8 ± 0.49
	5	5	5.01	100.3		5.04	100.8	
	5	5	5.07	101.4		5.07	101.4	
120 %	5	6	6.00	500.5	99.9 ± 0.34	5.95	99.3	99.6 ± 0.43
	5	6	5.97	497.7		5.97	99.5	
	5	6	6.01	500.9		6.00	100.1	

Table 6: Percent degradation of Metformin with retention time of the degradation products

Sr. No.	Conditions	Retention time of Metformin / degradation products (min)	% Degradation of Metformin (n=5)	% Degradation of Metformin in synthetic mixture (n=5)
1	Untreated stock solution (10µg/ml)	3.78	-	-
2	Acid hydrolysis	4.51, 7.96	14.52	13.90
3	Alkali hydrolysis	2.53,3.35,5.12	18.99	18.13
4	Oxidative degradation	3.03,5.35	20.90	20.65
5	Thermal degradation	2.64, 4.66	14.05	14.28
6	Photolytic degradation	2.44, 4.31	18.87	18.23

Table 7: Percent degradation of Dapagliflozin with retention time of the degradation products

Sr. No.	Conditions	Retention time of Dapagliflozin / degradation products (min)	% Degradation of Dapagliflozin (n=5)	% Degradation of Dapagliflozin in synthetic mixture (n=5)
1	Untreated stock solution (10µg/ml)	5.74	-	-
2	Acid hydrolysis	4.12,7.29	18.02	18.93
3	Alkali hydrolysis	2.83, 5.00	15.21	13.60
4	Oxidative degradation	2.90, 5.14	16.04	16.85
5	Thermal degradation	2.90, 5.12	10.03	9.00
6	Photolytic degradation	2.82, 4.97	16.27	16.79

Percentage recovery was in the range of 99.9 - 100.8 % for Metformin and 99.9 - 100.8 % for Dapagliflozin. LOD was 0.26 µg/ml and 0.41 µg/ml for Metformin and Dapagliflozin respectively. LOQ was 0.79 µg/ml and 1.24 µg/ml for Metformin and Dapagliflozin respectively. The method was robust and % RSD values < 2% with the deliberate changes in the composition of mobile phase, changes in the pH or change in the flow rate. Applicability of the proposed method was evaluated by analyzing a synthetic laboratory mixture and the assay results were 99.0 % and 99.8 % of Metformin and Dapagliflozin respectively in synthetic laboratory mixture.

Establishment of stability indicating method for assessment of degradation behavior

The stressed samples were assayed using developed RP-HPLC method. Following degradation behavior was observed under different stress conditions for the high-performance liquid chromatography studies on the combination of Metformin and Dapagliflozin [Table 6-7].

Significant degradation was observed in the presence of acidic, basic, neutral oxidative and photolytic stress conditions for Metformin and Dapagliflozin respectively (n=5). Percentage Degradation for the standard drug was 14%, 18%, 20%, 14% and 18% for Metformin and 18%, 15%, 16%, 10% and 16% for Dapagliflozin in the presence of acidic, basic, thermal, oxidative and photolytic degradation

respectively. Percentage Degradation for the Metformin in synthetic laboratory mixture was 13%, 18%, 20%, 14% and 18% in the presence of acidic, basic, thermal, oxidative and photolytic degradation respectively. Percentage degradation for Dapagliflozin in synthetic laboratory mixture was 18%, 13%, 16%, 9% and 16% in the presence of acidic, basic, thermal, oxidative and photolytic degradation respectively. The percent degradation was calculated by the formula: % degradation = (Average peak area of untreated stock solution – average peak area of stock solution under specific degradation condition)/(average peak area of untreated stock solution) x 100)

CONCLUSION

Proposed reversed phase high performance liquid chromatographic method was able to successfully separate and quantify Metformin and Dapagliflozin simultaneously in the presence of their degradation products. Degradation peaks were not interfering with the peaks of the analytes of interest. This implies the stability indicating nature and specificity of the method. The developed validated stability indicating RP-HPLC method is simple, precise, accurate, robust and reproducible resolving all the degradation products from the analytes of interest. Thus, the proposed method can be applied for the determination of Metformin and Dapagliflozin in bulk drug, pharmaceutical pre-formulation and formulations development studies in pharmaceutical research laboratories.

Future directions: This method can be successfully used to collect the peaks and identify the degradation products of Metformin and Dapagliflozin using mass spectrometry.

Acknowledgments: The authors wish to thank Dr. Rajshree Mashru and Dr. Jolly Parikh for providing their valuable scientific inputs.

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Source of Support: Nil, **Conflict of Interest:** None.