Original Research Article **Analytical Chemistry**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD WITH PHOTODIODE ARRAY DETECTION FOR THE SIMULTANEOUS ESTIMATION OF HYPOGLYCEMIC AGENTS, DAPAGLIFLOZIN AND METFORMIN

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ABSTRACT

The aim of the present study was to develop and validate a stability indicating reverse phase high performance liquid chromatographic (RP-HPLC) method for simultaneous quantitative analysis of dapagliflozin and metformin in bulk and tablets. Dapagliflozin, metformin and stress degradation products were eluted on the Supelco C8, 250 mm x 4.6 mm, 5 µm analytical column with a mobile phase consisting of 0.1 M dipotassium hydrogen phosphate, acetonitrile and methanol (60:30:10 v/v/v), pumped at 1.2 mL/min flow rate. The column temperature was set 30°C and 10 µL of the samples were injected. Detection was done at 285 nm. According to ICH guidelines, the developed RP-HPLC method was validated. The retention times of dapagliflozin and metformin were 2.847 min and 3.804 min, respectively. Linearity was observed in the concentration range of 2-6 µg/mL (R^2 = 0.9999) for dapagliflozin and 200-600 µg/mL *(R^2 = 0.9998)* for metformin. The limit of detection for dapagliflozin and metformin were found to be 0.004 µg/mL and 0.272 µg/mL, respectively. The percent recovery and percent relative standard deviation for the selected drugs were in the range of 99.00%-99.82% and 0.098%-0.291%, respectively. Non interference of peaks from stress degradation products in acidic, alkaline, oxidative, thermal and photolytic conditions demonstrated the stability indicating power of the method. The proposed RP-HPLC method can be used as a stability indicating method for the assay of dapagliflozin and metformin simultaneously in bulk and tablet dosage form.

KEY WORDS: ***Dapagliflozin, metformin, reverse phase high performance liquid chromatography, stability indicating method, stress degradation***

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Received on: 24-May-17
Revised and Accepted on: 04-Jul-17
DOI: http://dx.doi.org/10.22376/ijpbs.2017.8.3.p328-336

INTRODUCTION

Dapagliflozin is an antihyperglycemic agent belonging to gliflozin class of drugs.[1] Chemically, it is described as (2S,3R,4R,5S,6R)-2-[4-chloro-3-[(4-ethoxyphenyl) methyl] phenyl]-6-(hydroxymethyl)oxane-3,4,5-triol (Fig 1). In 2014, the US Food and Drug Administration approved dapagliflozin for glycemic control in adult patients with type II diabetes.[2] Dapagliflozin acts as selective inhibitor for sodium-glucose co-transport subtype 2 proteins.[3-5] These proteins are responsible for the reabsorption of glucose in the kidney. By inhibiting these proteins, dapagliflozin decreases blood sugar levels by causing the kidneys to eliminate more glucose in the urine. Metformin is an oral hypoglycemic agent belonging to the biguanides class of compounds. Chemically, metformin is described as 3-(diaminomethylidene)-1,1-dimethylguanidine (Fig 1). Metformin is prescribed for the management of non insulin dependent diabetes mellitus.[6,7] Metformin exerts hypoglycemic activity by decreasing hepatic production & intestinal absorption of glucose and improving insulin sensitivity. All the effects are mediated by the activation of enzyme adenosine monophosphate (AMP)-activated protein kinase by metformin.[8-10]

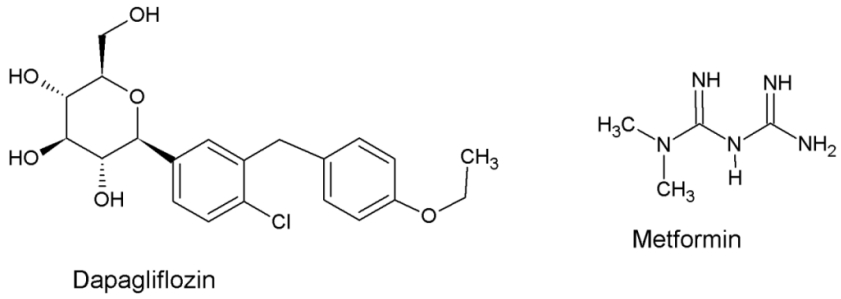

Figure 1
Chemical structures of the selected drugs

In 2014, the US Food and Drug Administration have approved the combination of dapagliflozin and metformin, along with diet and exercise, to improve blood glucose control in adults with type II diabetes.[11] An extensive literature survey was done and found that there were two UV spectrophotometric methods[12,13] and two RP-HPLC methods[14,15] for the determination of dapagliflozin and metformin simultaneously. In the present study, an attempt was made to develop a new stability indicating RP-HPLC method for simultaneous estimation of dapagliflozin and metformin in bulk and tablet dosage form with good sensitivity, selectivity, linearity, precision, accuracy and reproducibility.

MATERIALS AND METHODS

HPLC apparatus

Waters 2695 alliance HPLC system comprised an autosampler injector, binary pump and a Waters 2998 Photo Diode Array Detector coupled with Waters Empower2 software.

Materials

Metformin and dapagliflozin were obtained as gift sample from Lara Drugs Private Limited (Telangana, India). Xigduo XR tablets (Astra Zeneca Pharmaceuticals LP, Wilmington) were obtained from the local pharmacy market. Acetonitrile (HPLC grade) and methanol (HPLC grade) were supplied by Merck India Ltd., Mumbai, India. Analytical reagent grade dipotassium hydrogen orthophosphate, hydrogen peroxide, hydrochloric acid and sodium hydroxide were obtained from Sd. Fine Chemicals Ltd., Mumbai, India. Milli-Q water (Millipore, USA) was used in all experiments.

Chromatographic conditions

The chromatographic separation of metformin, dapagliflozin and their stress degradants were carried out on a Supelco C18 (250 mm × 4.5 mm i.d., particle size 5 µm). The mobile phase was a mixture of 0.1 M dipotassium hydrogen phosphate, acetonitrile and methanol (60:30:10, *v/v/v;* pH 7.5) delivered at a flow rate of 1.2 mL/min. The mobile phase was filtered through 0.45 µm pore size membrane filter and sonicated for 20 min. Analysis was performed at 30^{o}C temperature. The elution of metformin and dapagliflozin was detected by photodiode array detection. The chromatograms were recorded at 285 nm and the injection volume was 10 µL.

Stock and working solutions

Stock standard solution of dapagliflozin (0.1 mg/mL) and metformin (10 mg/mL) was prepared by dissolving 10 mg of dapagliflozin and 1000 mg of metformin in 100 mL of mobile phase in a 100 mL volumetric flask. This stock standard solution was used to prepare the working solutions at different concentrations (dapagliflozin - 2, 3, 4, 5 and 6 µg/mL: metformin – 200, 300, 400, 500 and 600 µg/mL). The stock and working standard solutions were stored in refrigerator until further use.

Calibration curve

Aliquots of the working standard solutions (10 µL) were injected into the HPLC system. The peak areas of dapagliflozin and metformin were determined using the chromatographic conditions described above. The preak area was plotted against the concentration of drug to construct the calibration curve. The y-intercept, slope and regression coefficient were calculated to statistically evaluate the linear relationship.

Determination of dapagliflozin and metformin in tablet dosage form

Ten tablets of Xigduo XR (each tablet labelled to contain 10 mg dapagliflozin and 1000 mg metformin) were powdered. An amount equivalent to 10 mg dapagliflozin and 1000 mg metformin was accurately weighed into a 100 mL volumetric flask and mixed with 30 mL of mobile phase. The solution was sonicated for 20 min and make up with mobile phase to obtain a final concentration of 0.1 mg/mL (dapagliflozin) and 10 mg/mL (metformin). The solution was filtered through a 0.45 µm pore size membrane filter.Aliquot of the above tablet sample stock solution were further diluted with the mobile phase to obtain final concentration of 4 µg/mL of dapagliflozin and 400 µg/mL of metformin. The resulting solution was then subjected to analysis by the proposed RP-HPLC method.

Stress testing

Stress testing was carried out to induce forced degradation, to identify the stability of the drugs and also to validate the specificity of the proposed RP-HPLC method. Forced degradation was performed by exposing tablet sample solution (metformin - 400 µg/mL and dapagliflizone - 4 µg /mL) to stress conditions of hydrolysis (acid & alkali), oxidation, photo and thermal.[16] The acid and alkali stress was performed with 0.1 N HCl and 0.1 N NaOH, respectively, and sonicated for 30 min at room temperature (25±2 °C). Oxidative stress was performed with 30 % hydrogen peroxide solution and sonicated for 30 min at room temperature. Photolytic and thermal stress studies were conducted by exposing the sample to direct sun light for up to 24 hr and to 105 ^{o}C for 30 min in oven, respectively. Stressed samples were analyzed by the proposed RP-HPLC. The metformin and dapagliflozin peaks were checked for the retention times, peaks interference and spectra purity.

RESULTS AND DISCUSSION

Method development

Supelco C8 and C18 column with different temperatures were tried. The Supelco C18 column (250 mm × 4.5 mm, 5 µm) at 30^{o}C temperature was found to be apt for the separation of metformin and dapagliflozin efficiently. Various ratios of 0.1 M dipotassium hydrogen phosphate, acetonitrile and methanol with different flow rates and pH values were tested using a Supelco C18 (250 mm × 4.5 mm, 5 µm) column. Results were evaluated in terms of peak response, resolution, peak symmetry, selectivity and analysis time for drugs. The mobile phase with a composition of 60% 0.1 M dipotassium hydrogen phosphate, 30% acetonitrile and 10% methanol (*v/v/v*) with the flow rate of 1.2 mL/min and pH 7.5 exhibited the appropriate separation of metformin and dapagliflozin with good peak shape and resolution. The detection wavelength for dapagliflozin and metformin was obtained using the PDA detector. A wavelength of 285 nm was selected for the simultaneous determination of dapagliflozin and metformin with good sensitivity. The typical chromatogram of dapagliflozin and metformin using the optimized chromatographic conditions is shown in Fig 2. The proposed method permitted adequate resolution of the dapagliflozin and metformin within reasonable run time (6 min). Dapagliflozin and metformin were eluted at 2.847 min and 3.804 min, respectively.

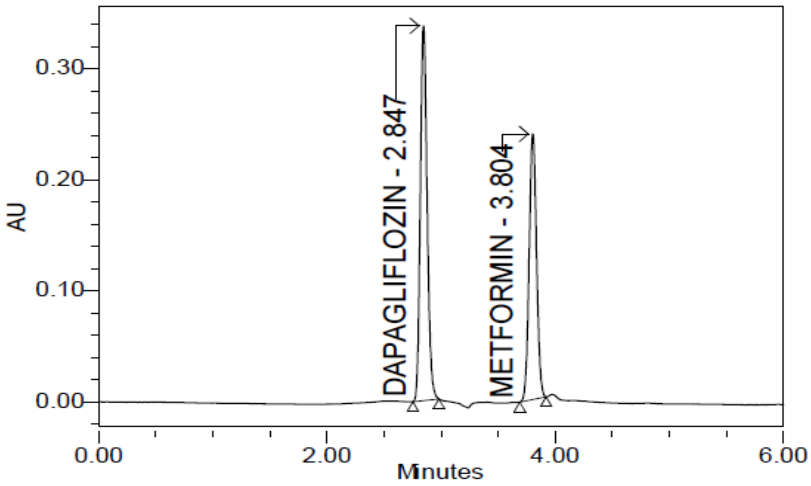

Figure 2
Chromatogram of dapagliflozin and metformin with optimized chromatographic conditions

Method validation

The developed RP-HPLC method was validated regarding system suitability, selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, robustness and specificity according to the International Conference on Harmonization.[17]

System suitability

To evaluate the system suitability of the developed RP-HPLC method, five replicate analyses were done at a concentration of 4 µg/mL dapagliflozin and 400 µg/mL metformin. The system suitability parameters (% RSD of retention time, % RSD of peak area, USP plate count and USP tailing factor) were calculated and compared with the accepted criteria (Table 1). The results reveal the method suitability.

Table 1
System suitability parameters

Parameters	Metformin			Dapagliflozin			Recommended limit
	Value*	SD	RSD (%)	Value*	SD	RSD (%)	
Retention time	3.807	0.0035	0.093	2.852	0.0044	0.156	RSD ≤2
Peak area	1074166	2777.404	0.259	1391792	5491.485	0.395	RSD ≤2
USP resolution	8.218	0.0645	0.786	-	-	-	> 1.5
USP plate count	16276	193.703	0.190	11053	138.673	0.255	> 2000
USP tailing factor	1.044	0.0054	0.525	1.162	0.0044	0.385	≤ 2

Average of five values

Selectivity

Selectivity was assessed by evaluating the chromatograms of mobile phase blank, placebo blank, working standard solution and tablet sample solution. The solutions of working standard and tablet sample were prepared at a concentration of 4 µg/mL dapagliflozin and 400 µg/mL metformin. The solutions of placebo blank, mobile phase blank, working standard and tablet sample were injected into the HPLC system. The chromatograms of placebo blank and mobile phase did not show any peaks (Fig 3). The chromatogram of tablet sample did not show any peaks other than that of metformin and dapagliflozin (Fig 3). The retention time of metformin and dapagliflozin in chromatograms of working standard solution and tablet sample solution are same (Fig 3). The results confirmed the specificity of the developed RP-HPLC method.

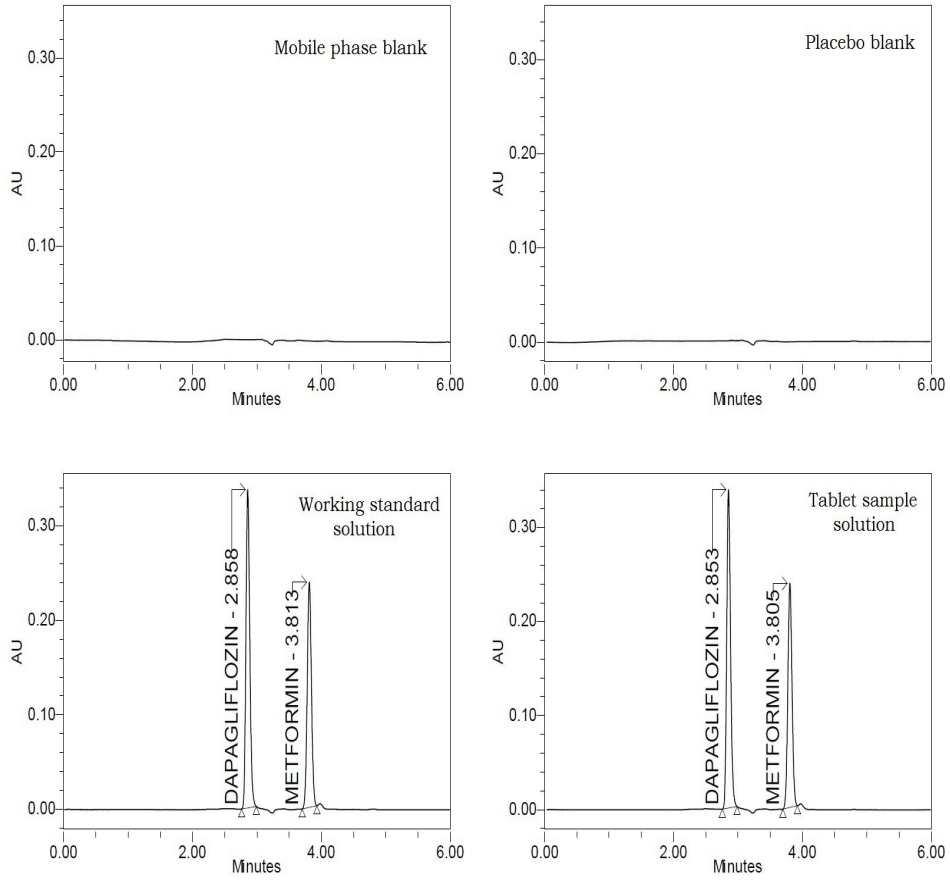

Figure 3
Chromatograms of selectivity studies

Linearity

The linearity of the developed RP-HPLC method was obtained in the concentration range of 2-6 µg/mL for dapagliflozin and 200-600 µg/mL for metformin. The regression equations for the calibration curve were:

Metformin: y = 2694 x – 1680 (R^2 = 0.9998)

Dapagliflozin: y = 34790 x + 336.0 (R^2 = 0.9999)

Where y = peak area; x = concentration of drug in µg/mL; R^2 = Regression coefficient

The results demonstrated that the linearity of the method as satisfactory.

LOD and LOQ

The LOD and LOQ are defined as a signal-to–noise of more than three and ten-fold, respectively. The LOQ for dapagliflozin and metformin in this method were found to be 0.014 µg/mL and 0.907 µg/mL, respectively. The LOD of dapagliflozin and metformin were found to be 0.004 µg/mL and 0.272 µg/mL, respectively.

Precision

Method precision was evaluated by injecting five independent working standard solutions with concentration 4 µg/mL of dapagliflozin and 400 µg/mL of metformin. The percentage relative standard deviation (% RSD) of peak area response was determined. % RSD values for dapagliflozin and metformin were found to be 0.098% and 0.290%, respectively. The results (Table 2) of precision testing proved that the developed method is precise.[17]

Table 2
Precision and accuracy

Sample No.	Dapagliflozin		Metformin	
	Peak area (mAU)	Recovery (%)	Peak area (mAU)	Recovery (%)
1	1395556	99.37	1073942	99.68
2	1394761	99.31	1078341	100.1
3	1397868	99.53	1071294	99.43
4	1394339	99.28	1071016	99.41
5	1395322	99.35	1071352	99.44
Mean*	**1395569**	**99.36**	**1073189**	**99.61**
SD	**1370.432**	**0.097**	**3114.387**	**0.290**
RSD	**0.098**	**0.098**	**0.290**	**0.291**

Average of five values

Accuracy

For evaluating accuracy of the method, dapagliflozin and metformin concentration of 4 µg/mL and 400 µg/mL level was determined in five replicates. Table 2 illustrates the accuracy data of the method. The percent recovery was calculated and found to be 99.36% and 99.61% for dapagliflozin and metformin, respectively. The results confirmed the accuracy of the proposed method.Accuracy of the method was further determined by spiking preanalyzed tablet sample with known amounts of dapagliflozin and metformin at three concentration levels.[17] The samples were once again analyzed by the proposed method under optimized conditions. The mean recovery rates were found in the range 99.00% to 99.46% for dapagliflozin and 99.70% to 99.82% for metformin (Table 3). The results showed that the presence of excipients in the tablet does not interfere in the determination of dapagliflozin and metformin.

Table 3
Recovery of dapagliflozin and metformin

Spiked Level	Dapagliflozin				Metformin			
	Added (µg/mL)	Found (µg/mL)	Recovery (%)	Mean* (%)	Added (µg/mL)	Found (µg/mL)	Recovery (%)	Mean* (%)
50%	2	1.980	99.00	99.00	200	199.587	99.79	99.73
	2	1.979	98.95		200	199.409	99.70	
	2	1.981	99.05		200	199.410	99.71	
100%	4	3.986	99.65	99.46	400	398.710	99.68	99.70
	4	3.974	99.35		400	397.280	99.32	
	4	3.975	99.38		400	400.430	100.11	
150%	6	5.977	99.62	99.29	600	600.760	100.13	99.82
	6	5.949	99.15		600	598.320	99.72	
	6	5.947	99.12		600	597.610	99.60	

Average of three values

Robustness

To evaluate the robustness of the proposed RP-HPLC method, the effect of minor variations in the flow rate (± 0.1 mL) and column temperature (± 5°C) on system suitability parameters were observed. Robustness was studied using working standard solution containing dapagliflozin and metformin at a concentration of 4 µg/mL and 400 µg/mL, respectively. The results are shown in Table 4. There was no significant change in the system suitability parameters, thus established the robustness of the proposed RP-HPLC method.

Table 4
Results of method Robustness

Parameter	Dapagliflozin			Metformin		
	USP Tailing	USP plate count	USP resolution	USP Tailing	USP plate count	USP resolution
Flow rate 1.0 + 0.1 mL/min	1.17	10889	-	1.04	16527	8.27
Flow rate 1.0 – 0.1 mL/min	1.16	11146	-	1.08	16314	8.21
Temperature 30 + 5 °C	1.16	11093	-	1.05	16229	8.24
Temperature 30 - 5 °C	1.15	11210	-	1.07	16323	8.11

Specificity

Forced degradation was performed to evaluate the specificity and stability indicating properties of the developed RP-HPLC method, by exposing tablet sample to different stress conditions like hydrolysis (acid & alkali), oxidation, photo and thermal stresses. The degradation results are summarized in Table 5. Upon treatment of dapagliflozin and metformin under different stress conditions, it was found that both the drugs were degraded in all the stress conditions applied. From the percentage of degradation values, it was indicated that the dapagliflozin is less stable than metformin. In all the degradation conditions two peaks, in addition to dapagliflozin and metformin peaks, were detected. From the results, it was observed that there is no interference between the peaks of dapagliflozin, metformin and degradation products under the various stress conditions (Figs 4-8). The peak purity tool was applied to confirm 100% purity for dapagliflozin and metformin peaks in all cases. The peaks of dapagliflozin and metformin were pure because purity threshold was greater than purity angle under all the forced tests. The results showed that dapagliflozin and metformin peaks are free of coeluting degradation products.

Table 5
Summary of degradation studies of dapagliflozin and metformin

Stress condition	Peak area (mAU)	Recovery (%)	Degradation (%)	Purity threshold	Purity angle
Dapagliflozin					
Acid induced	1191919	84.87	15.13	0.370	0.241
Alkali induced	1177716	83.86	16.14	0.371	0.244
Oxidative	1192189	84.89	15.11	0.384	0.244
Thermal	1192280	84.89	15.11	0.390	0.248
Photo	1191517	84.84	15.16	0.377	0.245
Metformin					
Acid induced	998763	92.70	7.30	0.375	0.236
Alkali induced	990644	91.95	8.05	0.393	0.238
Oxidative	998149	92.64	7.36	0.407	0.235
Thermal	997657	92.60	7.40	0.405	0.239
Photo	991688	92.04	7.96	0.402	0.234

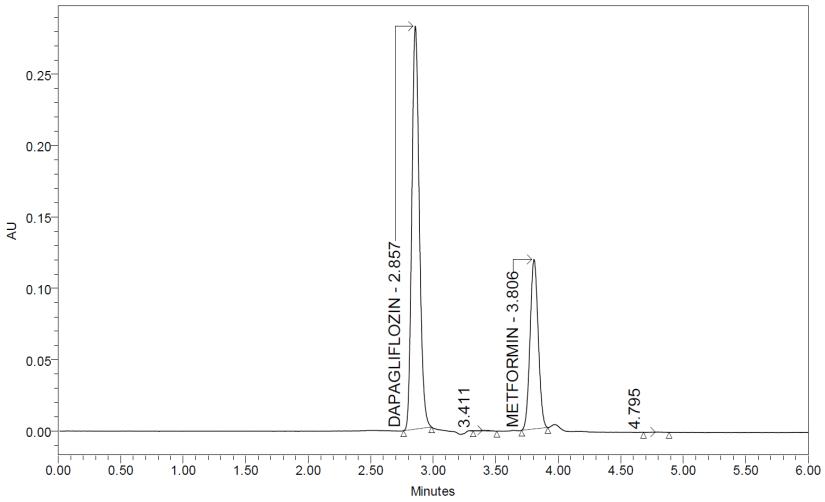

Figure 4
Dapagliflozin and metformin in 0.1 N HCl after 30 min at room temperature

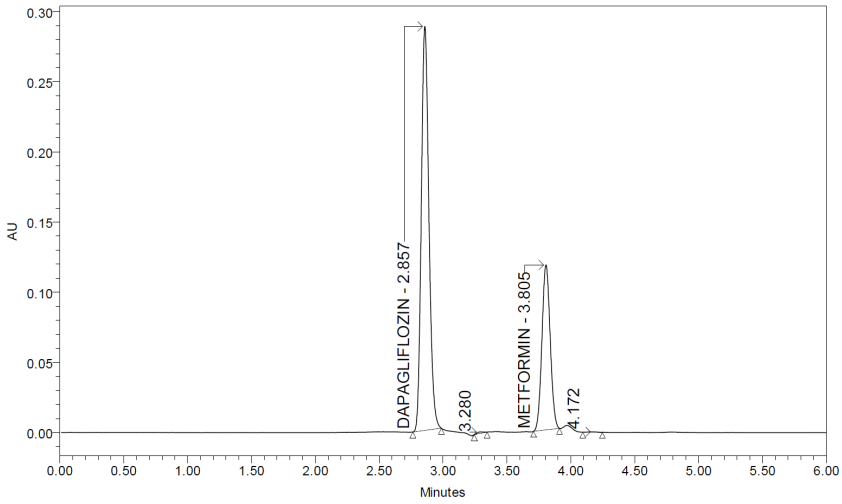

Figure 5
Dapagliflozin and metformin in 0.1 N NaOH after 30 min at room temperature

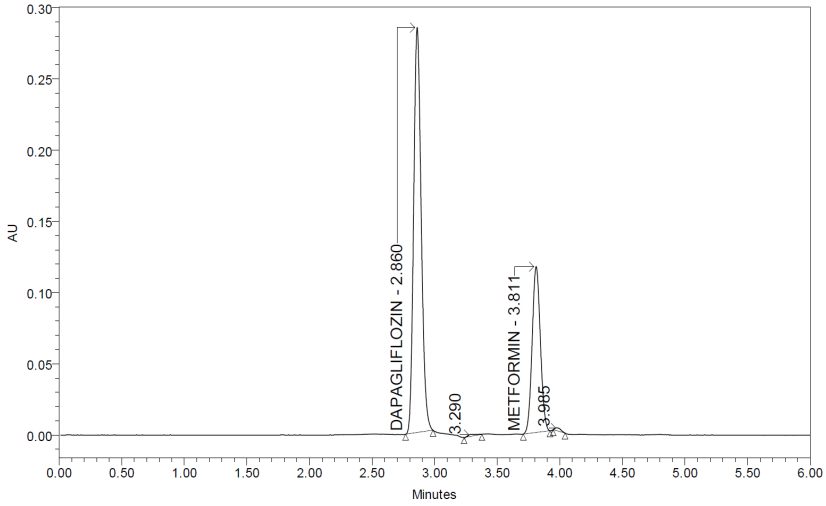

Figure 6
Dapagliflozin and metformin in 30% H_2O_2 after 30 min at room temperature

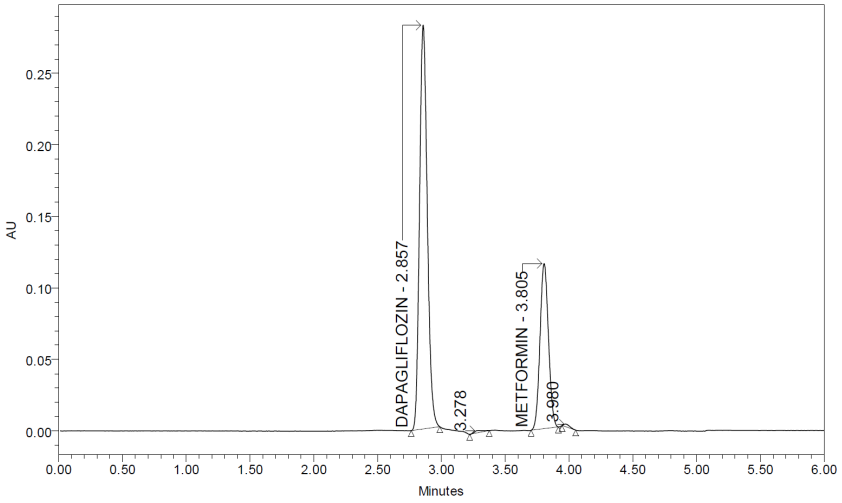

Figure 7
Dapagliflozin and metformin after exposure 105 ^{o}C for 30 min

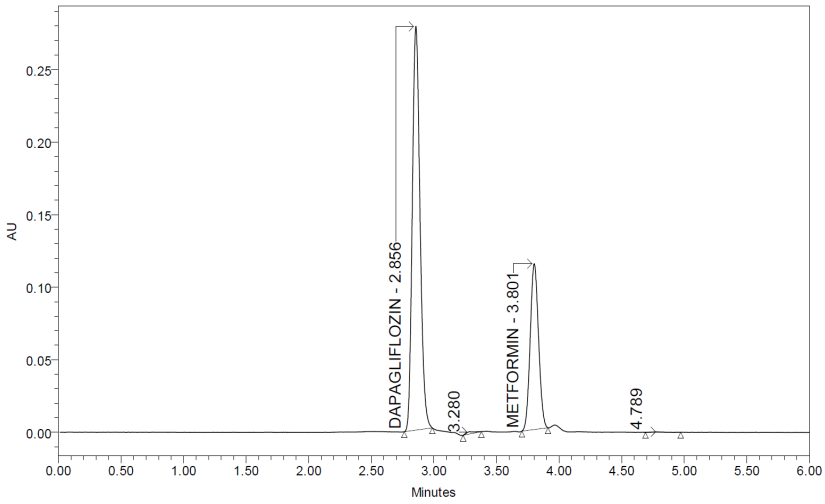

Figure 8
Dapagliflozin and metformin after 24 hours of exposure to sunlight

Comparison among the proposed RP-HPLC method and other reported methods

The present method for the simultaneous determination of dapagliflozin and metformin was compared with other reported UV spectrophotometric and HPLC methods as shown in Table 6. Though the UV spectrophotometric methods are simple[12,13] and sensitive[13], they are less selective and are not applied to tablet dosage form. In Jani et al[12] UV spectrophotometric method, LOD and LOQ were not reported. The proposed method have broad range of linearity[12-15], more sensitive[14,15], more precise and accurate[12-15] than the reported methods. The volume of sample used for single analysis by the proposed method (10 µL) is less than the reported methods (≥ 20 µL).[12-15] The less run time makes the proposed RP-HPLC method more rapid than reported HPLC methods.[14,15]

Table 6
Comparison among the proposed method and other methods in the literature applied to assay of dapagliflozin and metformin

Method	Drug	Detection wavelength (nm)	Run Time (min)	Linearity (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)	RSD (%)	Recovery (%)	Reference
UV spectro photometry	Met	225 & 237	-	25-125	NR	NR	0.484-0.641	100.48-102.96	Jani et al. [12]
	Dapa	225 & 237		0.5-2.5	NR	NR	0.551-0.582	99.10-102.40	
UV spectro photometry	Met	272	-	25-125	0.013	0.041	0.235-0.399	99.34-99.95	Jani et al. [13]
	Dapa	235		0.5-2.5	0.009	0.039	0.760-0.929	98.15-99.66	
RP-HPLC	Met	240	7	85-510	1.32	3.95	0.52-0.83	99.66-100.23	Mohammad & Gowri [14]
	Dapa	240		0.5-3.0	0.43	1.43	0.26-0.37	99.61-100.38	
RP-HPLC	Met	240	7	85-510	2.469	2.468	1.22	99.83-100.65	Shyamala et al [15]
	Dapa	240		0.5-3.0	3.650	3.649	0.98	99.48-100.54	
RP-HPLC	Met	285	6	200-600	0.272	0.907	0.290	99.70-99.82	Proposed
	Dapa	285		2-6	0.004	0.014	0.098	99.00-99.46	

Met - metformin; Dapa - dapagliflozin; NR - not reported

CONCLUSION

A sensitive, precise and accurate stability indicating RP-HPLC method with a photodiode array detector has been proposed for the simultaneous analysis of dapagliflozin and metformin. The developed RP-HPLC method was validated as per ICH guidelines. The validation results showed that the proposed method was rapid, sensitive, precise, accurate and selective than the reported methods. The proposed method provides a stability-indicating assay for the quantification of dapagliflozin and metformin in bulk powder and tablets, without interference from the common excipients used in the preparation of tablets and in the presence of acidic, alkaline, oxidative, thermal and photolytic degradation products. The degradation products were well separated from the dapagliflozin and metformin signifying the stability indicating nature of the method. Hence the developed RP-HPLC method is a stability indicating assay that can be used for the routine analysis of dapagliflozin and metformin in bulk and tablets without any interference.

ACKNOWLEDGEMENTS

The authors are thankful to Department of Chemistry, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, India for support and encouragement.

CONFLICT OF INTEREST

Conflict of interest declared none.

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We sincerely thank the above reviewers for peer reviewing the manuscript