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**CHROMATOGRAPHIC SIMULTANEOUS QUANTIFICATION OF DAPAGLIFLOZIN
AND METFORMIN HYDROCHLORIDE IN PRESENCE OF THEIR DEGRADATION
PRODUCTS**

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ABSTRACT

A reversed phase high-performance liquid chromatographic (RP-HPLC) method was adopted and validated as a stability indicating method for the simultaneous quantification of dapagliflozin (DAPA) and metformin (MET) hydrochloride in presence of their degradation products. The degradation process was carried out under acidic, basic, oxidative and thermal conditions, as recommended by the International Conference on Harmonization (ICH)-guidelines. Also, the stability of either the bulk or tablet forms was studied under the storage conditions indicated by the ICH-guidelines (temperature of 40°C and relative humidity of 75%, for 6 months). Validation parameters such as linearity, accuracy, precision, specificity, limits of detection and quantification (LOD & LOQ) were determined. The best chromatographic separation pattern was acquired on using Hypersil™ ODS C₁₈ column (150 x 4.6 mm, 5 μm) as a stationary phase and 0.05 M potassium dihydrogen phosphate buffer (adjusted to pH 4.6 using orthophosphoric acid):acetonitrile:methanol (5:4:1, *by volumes*), as a mobile phase. It was pumped using an isocratic mode with flow rate of 0.5 mL/min. and UV detection at 236 nm. The calibration graph was linear in the range of 0.5 -20 μg/mL, for DAPA and 50 - 550 μg/mL, for MET. The

proposed method is accurate, sensitive and precise, so it can be successfully adopted for the reliable simultaneous determination of DAPA and MET in either their bulk and tablet forms.

Keywords: Dapagliflozin; Metformin; Stability; Quantification

1. INTRODUCTION

Diabetes can be considered as a group of metabolic disorders which is characterized by elevated blood glucose sugar for a long time [1]. In January, 2014, Food and drug administration (FDA) approved dapagliflozin (DAPA) for glycemic control in adults suffering from type 2 diabetes and in the same year, FDA approved its combination with metformin hydrochloride (MET). DAPA, (2S,3R,4R,5S,6R)-2-[4-chloro-3-[(4-ethoxyphenyl)methyl]phenyl]-6-(hydroxymethyl)oxane-3,4,5-triol, exerts its action by inhibiting subtype 2 of the sodium-glucose transport proteins (SGLT2) which are responsible for most of the glucose reabsorption in the kidney. Blocking this transporter mechanism causes blood glucose to be eliminated through the urine leading to reduction of blood glucose level [2].

MET hydrochloride, 3-(diamino methylidene)-1, 1- dimethyl guanidine; hydrochloride, is an orally administered biguanide which exerts its effect in lowering blood glucose level by not only reducing hepatic glucose production and

gluconeogenesis, but also by enhancing peripheral insulin sensitivity [3,4].

The literature review revealed that, there are few analytical methods used for DAPA determination [5-7]. On the other hand, there are many methods used for MET quantification either alone or together with other drugs [8-13]. In an attempt to study the stability of the studied drugs, Yunoos and Sankar [14] determined DAPA and MET under various stress conditions using HPLC technique but the resolution of the separated drugs either from each other or from their degradation products was poor. Also, the linearity range for DAPA was very narrow. The variations in experimental conditions in the work of Yunoos and Sankar [14] were not cancelled specially during the setting of calibration curves, where the peak areas were used directly without applying the internal or external standard methods. Moreover, they do not study the effect of storage conditions on the stability of the studied drugs in their dosage form, which can give an integrated view for the stability pattern of the studied drugs. So, the main goal of the present work is to establish a novel chromatographic

method that can simultaneously quantify DAPA and MET either in presence of each other or in presence of their degradation products with high resolution, acceptable sensitivity and optimum linearity. Also, this work aims to study the effects of the storage conditions on the stability of the studied drugs according to the ICH-guidelines.

2. EXPERIMENTAL

2.1. Chemicals and reagents

Standard DAPA and MET hydrochloride were purchased from Cayman chemical company, Ann Arbor, United States of America (USA; their purities were certified to be 99.99% and 100.02%, respectively. Methanol, orthophosphoric acid and acetonitrile of HPLC grade were supplied by Sigma Aldrich, St. Louis, USA. Distilled water of HPLC grade was supplied by Merck Millipore, Darmstadt, Germany. Potassium dihydrogen phosphate was obtained from (Adwic, Cairo-Egypt).

2.2. Pharmaceutical formulation

FARIXIA[®] tablets, each tablet contains DAPA propanediol monohydrate equivalent to 10 mg DAPA. XIGDUO[®] XR film coated tablets, each tablet contains DAPA propanediol monohydrate equivalent to 5 mg DAPA and 850 mg MET hydrochloride. They were manufactured by AstraZeneca, Ontario,

Canada. Both tablet forms were procured from the US market.

2.3. Instrumentation

High performance liquid chromatograph consisted of a binary pump (Waters, 1525), a UV-visible wavelength detector (Waters, 2489) and an auto-sampler (Waters, 2707). The chromatograph is a product of Waters, Massachusetts, USA.

Stable Climate II stability chamber manufactured by Cincinnati Sub-Zero, 12011, Mosteller Road, Sharonville, OH 45241, USA.

2.4. Standard solutions

DAPA stock standard solution (0.5 mg/mL) was prepared by accurate weighing and transferring of 50 mg of pure DAPA into 100-mL measuring flask. The drug was dissolved by aid of a vortex mixer in 20 mL methanol then the volume was completed to the mark using the same solvent.

DAPA working standard solution (50 µg/mL) was prepared by accurate dilution of 5 mL of the stock standard solution (0.5 mg/mL) into 50-mL measuring flask using methanol as a diluting solvent.

MET standard solution (5 mg/mL) was prepared by accurate weighing and transferring of 250 mg of pure MET into 50-mL measuring flask. The drug was dissolved by aid of a vortex mixer in 20 mL distilled

water then the volume was completed to the mark using the same solvent.

2.5. Degradation procedure

2.5.1. Acidic degradation

Mixing of 0.2 mL of DAPA working standard solution (50 µg/mL) and 0.2 mL of MET standard solution (5 mg/mL) with 2 mL 2N hydrochloric acid solution was done. Refluxing of the mixture was carried out for 30 minutes at 60°C and then neutralization of the solution was done using 2 ml of 2N sodium hydroxide solution. The resultant solution was diluted with methanol in a 10 mL volumetric flask then it was chromatographed to monitor the degradation process.

2.5.2. Basic degradation

Mixing of 0.2 mL of DAPA working standard solution (50 µg/mL) and 0.2 mL of MET standard solution (5 mg/mL) with 2 mL 2N sodium hydroxide solution was done. Refluxing of the mixture was carried out for 30 minutes at 60°C and then neutralization of the solution was done using 2 mL of 2N hydrochloric acid solution. The resultant solution was diluted with methanol in a 10 mL volumetric flask then it was chromatographed to monitor the degradation process.

2.5.3. Oxidative degradation

Mixing of 0.2 mL of DAPA working standard solution (50 µg/mL) and 0.2 mL of MET standard solution (5 mg/mL) with 1 mL 20% hydrogen peroxide was carried out. The resultant solution was kept for 30 minutes at 60°C then the solution was diluted with methanol in a 10 mL volumetric flask then it was chromatographed to monitor the degradation process.

2.5.4. Thermal effect

Mixing of 0.2 mL of DAPA working standard solution (50 µg/mL) and 0.2 mL of MET standard solution (5 mg/mL) with 2 mL distilled water was performed. The resultant solution was refluxed on a water bath for 6 hrs at 60°C then the solution was diluted with methanol in a 10 mL volumetric flask then it was chromatographed to monitor the degradation process.

2.5.5. Stability of the dosage forms under storage conditions

Five FARIXIA[®] tablets labelled to contain DAPA propanediol monohydrate equivalent to 10 mg and five XIGDUO[®] XR film coated tablets labelled to contain DAPA propanediol monohydrate equivalent to 5 mg DAPA and 850 mg MET hydrochloride tablets were separately, stored for six months in Stable Climate[®] II stability chamber under the conditions of temperature/relative humidity (40 °C/75%).

2.6. Optimization of chromatographic conditions

The chromatographic conditions were optimized to get the best separation pattern of DAPA and MET from each other and from their degradation products. Many stationary and mobile phases were tried to carry out this task.

2.7. Method validation

The developed analytical method was fully validated according to ICH-Q2B guidelines [15].

2.7.1. Linearity

Aliquots of DAPA working standard solution (50 µg/mL) and MET standard solution (5 mg/mL) equivalent to 5 – 250 µg DAPA and 500 – 5500 µg MET were accurately and separately transferred into two groups of 10-mL volumetric flasks and the volume of each was completed to the mark with the methanol to obtain concentration ranges of 0.5 – 25 µg/mL and 50 – 550 µg/mL for DAPA and MET, respectively. Conditioning and pre-washing of the stationary phase was performed by passing about 60-70 mL of the mobile phase.

Samples were then chromatographed using Hypersil™ ODS C₁₈ column (150 x 4.6 mm, 5 µm) as a stationary phase. The mobile phase was 0.05 M potassium dihydrogen phosphate buffer (adjusted to pH 4.6 using

orthophosphoric acid):acetonitrile:methanol (5:4:1, by volumes). It was pumped using isocratic mode with flow rate of 0.5 mL/min. and UV detection at 236 nm. External standard method was adopted and so, peak area ratios were plotted against concentration to obtain the calibration graphs then the regression equations were computed.

2.7.2. Accuracy

It was assured by carrying out the previously mentioned procedures under linearity for determination of three different concentrations of pure DAPA (5, 10, 15 µg/mL) and MET (100, 200, 300 µg/mL). The concentrations were calculated from the corresponding regression equation.

2.7.3. Precision

It can be expressed as repeatability (intra-day) and intermediate precision (inter-day) as % relative standard deviation (% RSD), for a statistically significant number of experiments. So, three concentrations of DAPA (5, 10, 15 µg/mL) and MET (100, 200, 300 µg/mL) were analyzed three times within the same day (intra-day) or on three successive days (inter-day), then the results were documented as % RSD.

2.7.4. Specificity

Specificity can be determined by comparing the HPL chromatogram obtained for a mixture of the studied drugs together with the

commonly used excipients, against those obtained from the blank (excipients solution in methanol without drugs) [16].

2.7.5. Limits of detection and quantification (LOD & LOQ)

LOD can be defined as the lowest concentration of the analyte that the analytical method can reliably differentiate from the background. LOQ can be defined as the lowest concentration that can be quantified with acceptable accuracy and precision [16]. The LOD and LOQ were calculated as

$$\text{LOD} = 3.3 \sigma / S,$$

$$\text{LOQ} = 10 \sigma / S$$

Where, σ is the standard deviation of the lowest standard level and S is the slope of the standard curve.

2.7.6. Robustness

Robustness was assessed by evaluating the effect of minute variations on the proposed method. The conditions studied were the temperature variation by ± 5 °C and mobile phase composition with respect to acetonitrile by ± 5 mL acetonitrile per each 100 mL of the mobile phase.

2.7.7. System suitability

The system suitability parameters were evaluated according to tailing factor, number of theoretical plates (N), height equivalent to

theoretical plates (HETP), selectivity factor and resolution factor.

2.8. Analysis of pharmaceutical formulations (control and exposed to worm and humid conditions)

2.8.1. FARIXIA® tablets

Ten FARIXIA® tablets (five control and five exposed to worm and humid conditions) were separately weighed to find the average weight of a tablet then separately crushed, finely powdered and mixed well. Tablet powder from the control and exposed forms equivalent to 5 mg of DAPA was separately, transferred into two beakers of 250 mL capacity then; a suitable volume of methanol (40 mL) was added and stirred for about 20 minutes. Filtration was separately, carried out into two 100-mL volumetric flasks. The residue was washed with about 20 mL methanol (twice), and then the volume was completed to the mark with methanol and mixed well. Into 10 mL volumetric flask, 0.1 mL of the prepared solution was transferred then completing the volume to the mark was done using methanol. The prepared solution was chromatographed using the same conditions mentioned under linearity.

2.8.2. XIGDUO® XR film coated tablets

Ten XIGDUO® XR film coated tablets (five control and five exposed to worm and humid conditions) were separately weighed to find

the average weight of a tablet then separately crushed, finely powdered and mixed well. Tablet powder from the control and exposed forms equivalent to 5 mg of DAPA and 850 mg MET was separately, transferred into two beakers of 250 mL capacity then; a suitable volume of 1:1 distilled water / methanol (40 mL) was added and stirred for about 20 minutes. Filtration was separately, carried out into two 100 mL measuring flasks. The residue was washed with about 20 mL 1:1 distilled water / methanol (twice), and then the volume was completed to the mark with the same solvent and mixed well. Into 10 mL volumetric flask, 0.1 mL of the prepared solution was transferred then completing the volume to the mark was done using 1:1 distilled water / methanol. The prepared solution was chromatographed using the same conditions mentioned under linearity.

3. RESULTS AND DISCUSSION

There is no doubt that, stability of active pharmaceutical agents (APIs) in different conditions is a crucial factor in its efficacy and safety. This work can be regarded as a stability indicating one as it can determine DAPA and MET in presence of their degradation products. Also, this work can predict and suggest the suitable storage conditions for the studied dosage forms as we

studied the effect of temperature/relative humidity, according to the ICH-guidelines, on the stability of DAPA and MET.

The chromatographic parameters were optimized to get an optimum separation pattern for DAPA and MET from each other and from their degradation products. Different stationary and mobile phases were tried to achieve this task. The best Gaussian peak with ideal peak symmetry was obtained on using Hypersil™ ODS C18 column (150 x 4.6 mm, 5 μm) as a stationary phase and 0.05 M potassium dihydrogen phosphate buffer (adjusted to pH 4.6 using orthophosphoric acid):acetonitrile:methanol (5:4:1, by volumes) as a mobile phase. The mobile phase flow was operated using the isocratic mode with a rate of 0.5 mL/min. and UV detection at 236 nm. By adopting the described chromatographic conditions, DAPA was well resolved from MET at retention times 8.314 ± 0.05 min. (n=3) and 2.274 ± 0.04 min. (n=3) for DAPA and MET, respectively (Fig.1).

Also, the optimized chromatographic conditions were successfully applied for separation of the studied drugs from their degradation products under different conditions (Figs.2-5).

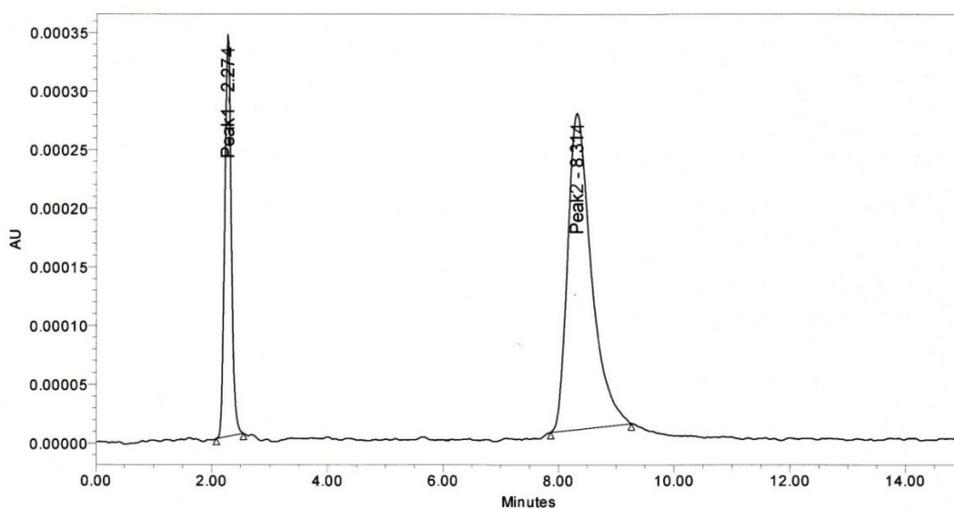


Fig. 1: Typical HPL chromatogram of standard DAPA (peak 2) and MET (peak 1)

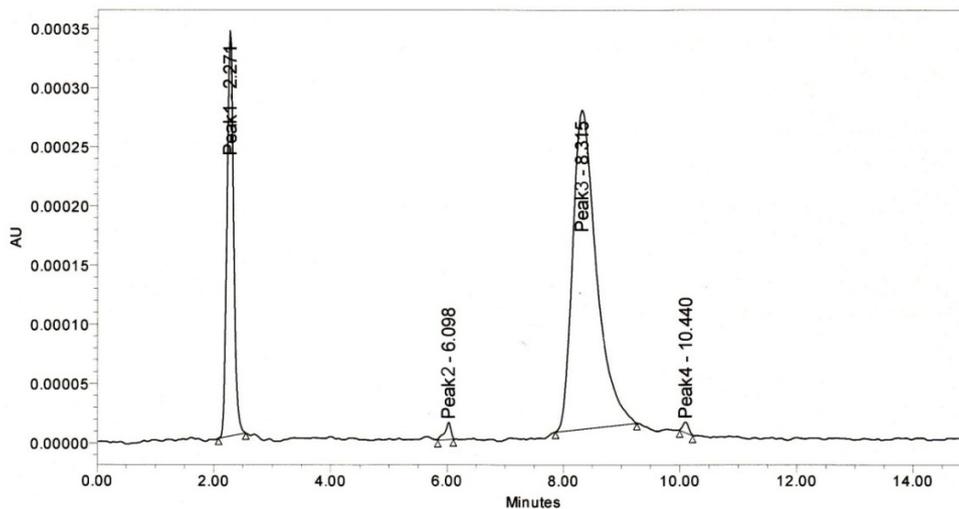


Fig. 2: HPL chromatogram of acidic degradation sample

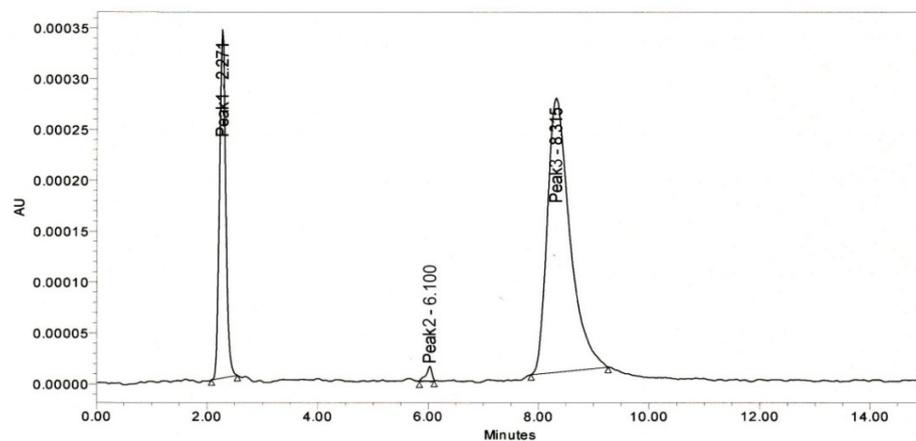


Fig. 3: HPL chromatogram of basic degradation sample

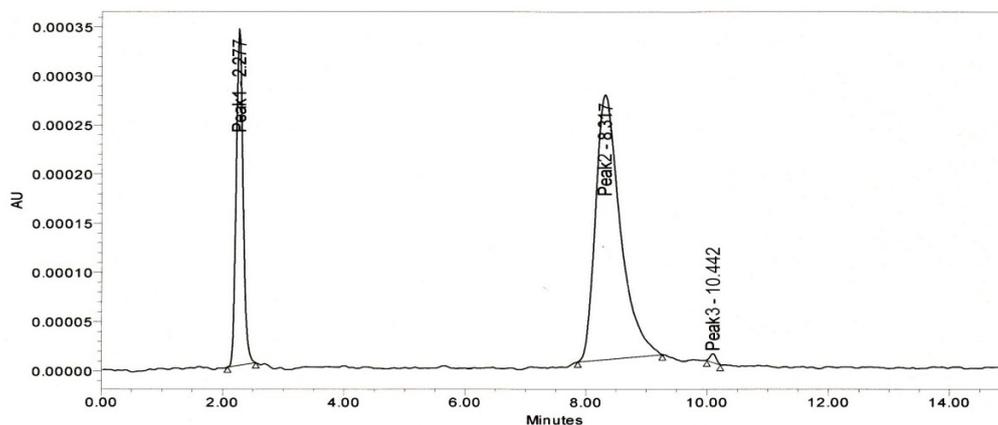


Fig. 4: HPL chromatogram of oxidative degradation sample

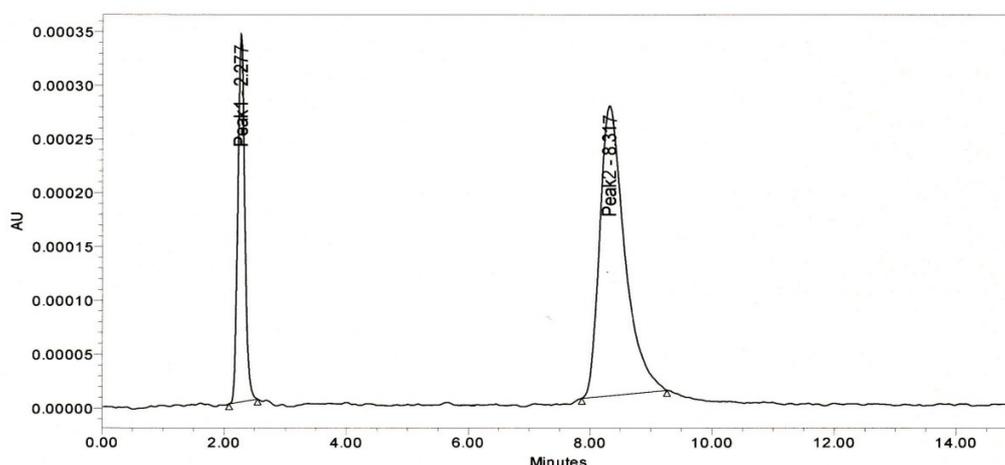


Fig. 5: HPL chromatogram of thermal degradation sample

3.1. Method validation

3.1.1. Linearity

Calibration standards at seven levels were prepared for each drug by suitable dilution of the corresponding standard solution (50 µg/mL for DAPA and 5 mg/mL for MET) to reach concentration range of 0.5 - 25 µg/mL, for DAPA and 50 – 550 µg/mL, for MET and each concentration was injected in triplicate. External standard method was used to eliminate any variations during the

preparation of the calibration standards, so the peak area ratios were plotted against the corresponding concentrations to obtain the calibration curve for each studied drug then the corresponding regression equation was computed to be:

$$P = 0.0996 C + 0.0003 \quad (r^2 = 0.9998) \text{ (for DAPA)}$$

$$P = 0.0033 C + 0.0021 \quad (r^2 = 0.9997) \text{ (for MET)}$$

Where, P is the peak area ratio and C is the concentration in µg/mL, Figs. 6, 7.

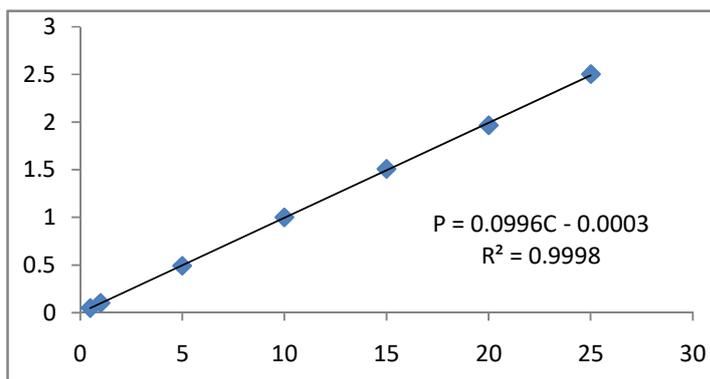


Fig. 6: Calibration curve of DAPA

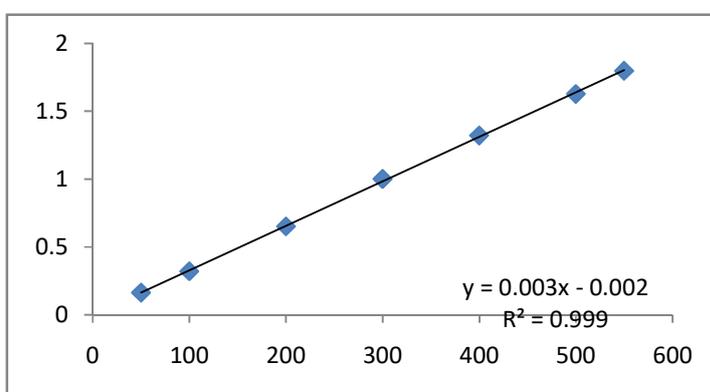


Fig. 7: Calibration curve of MET

3.1.2. Accuracy

The accuracy of the proposed method was validated by analyzing nine quality control samples of each studied drug representing three concentration levels covering the specified linearity ranges then calculating the recovery and percent of relative standard deviation (% RSD) which is considered satisfactory as it was less than 1% which confirm the accuracy of the developed method (Table 1).

3.1.3. Precision

The intraday and interday precisions were checked by analyzing three different

concentrations of each studied drug by adopting the proposed method, either in the same day or during three successive days. The % RSD values for intraday and interday precisions were less than 2% for the proposed methods, which confirm the good precision (Table 1).

3.1.4. Specificity

The proposed method was specific as none of the tried excipients interfered with the studied drugs so, the method was suitably applied for assaying the commercial products.

3.1.5. Limits of detection and quantification (LOD & LOQ)

The obtained values of LOD and LOQ confirmed the sufficient sensitivity of the proposed method (Table 1).

3.1.6. Robustness

It is a measure of the method capability to maintain unaffected by slight changes in its parameters. The studied parameters were the variation in temperature or mobile phase composition. The proposed method was not affected by the slight changes in its conditions where the % RSD values were less than 1% and so this confirms the robustness of the method (Table 1).

3.1.7. System suitability

System suitability parameters for the proposed method were studied, to evaluate the peak symmetry, column efficiency and resolution of separation between DAPA and MET. The studied parameters were tailing factor, number of theoretical plates (N), height equivalent to theoretical plates (HETP), selectivity factor (α) and resolution factor (R_s). The values of the studied parameters confirmed the excellent peak symmetry and high column efficiency, Table 2.

3.2. Analysis of pharmaceutical formulations

The proposed method was successfully adopted for the quantification of DAPA in the control and samples exposed to hot and humid conditions of FARIXIA[®] tablets and XIGDUO[®] XR film coated tablets. Also, the suggested method was used for determination of MET in the control and samples exposed to hot and humid conditions of XIGDUO[®] XR film coated tablets, Table 3. The non-exposed (control) tablet samples and also the samples exposed to temperature/relative humidity (40 °C/75%) showed acceptable recoveries by applying the proposed method which was within the acceptable limits of content uniformity. This may confirm the acceptable stability of the studied dosage forms when stored in the usual storage conditions suggested by the ICH- guidelines.

Also, the standard addition procedure was applied by spiking different known quantities of standard DAPA and MET to the tablet formulations to ensure the applicability and reliability of the proposed method. The results showed satisfactory recoveries of the standard added drugs by the proposed method (Table 3).

Table 1: Method validation parameters for determination of DAPA and MET by the adopted method

Parameter	DAPA	MET
Linearity		
Range	0.5 - 25 µg/mL	50 550 µg/mL
Slope	0.0996	0.0033
Intercept	- 0.0003	- 0.0021
r ²	0.9998	0.9997
Accuracy		
Mean ± S.D.*	100.15 ± 0.521	99.14 ± 0.712
Variance	0.271	0.507
%RSD	0.520	0.718
Precision		
Intraday precision	101.04 ± 0.914	102.14 ± 1.074
Interday precision	102.10 ± 1.084	99.01 ± 1.102
Robustness	99.12 ± 0.851	100.41 ± 0.418
	100.54 ± 0.475	101.51 ± 0.841
LOD	0.25 µg/mL	24.97 µg/mL
LOQ	0.50 µg/mL	48.99 µg/mL

*Standard deviation, †Average of three determinations, ^a Variation of temperature, ^b Variation of mobile phase composition

Table 2: Established system suitability parameters for the proposed method

Parameter	DAPA	MET	Significance
t _R (min.) [†]	8.314	2.274	-
Tailing factor	1.05	1.02	T=1 for a typical symmetric peak
Number of theoretical plates (N)	1105	2068	Increase with the increase in column efficiency
Height equivalent to theoretical plate (HETP)	0.013	0.007	Decrease with the increase in column efficiency
Selectivity factor (α)	-	3.66	High α values indicate good separating power
Resolution factor (R _s)	-	10.07	High R _s values indicate good separating power

[†]Average of triplicate runs

Table 3: Determination of DAPA and MET in FARIXIA[®] tablets and XIGDUO[®] XR film coated tablets and application of standard addition procedure by the proposed method

	Drug	Content uniformity		Standard addition	
		Mean ± SD *	% RSD	Mean ± SD*	% RSD
Non-exposed (control) FARIXIA [®] tablets containing 10 mg DAPA/tablet	DAPA	99.94± 0.512	0.512	101.04± 0.691	0.684
FARIXIA [®] tablets containing 10 mg DAPA/tablet exposed to temperature/relative humidity (40 °C/75%)	DAPA	99.11±0.841	0.849	-	-
Non-exposed (control) XIGDUO [®] XR film coated tablets containing 5 mg DAPA and 850 mg MET hydrochloride	DAPA	100.92± 0.758	0.751	102.01± 0.942	0.923
	MET	101.25± 0.711	0.702	99.89± 0.473	0.474
XIGDUO [®] XR film coated tablets exposed to temperature/relative humidity (40 °C/75%)	DAPA	100.25± 0.831	0.829	-	-
	MET	99.46± 0.769	0.773	-	-

*Average of three determinations

4. CONCLUSIONS

The proposed RP-HPLC method is a stability indicating one, which can be applied to monitor the stability pattern of DAPA and MET either in bulk or in their tablet forms. Also, the suggested method can be used as an analytical tool to suggest and recommend the suitable storage conditions for the studied dosage forms containing the studied drugs. Also, this method can be applied to confirm the content uniformity of the studied dosage forms.

The suggested method is simple, sensitive and selective having acceptable accuracy and precision. Also, the short analysis time makes the proposed method suitable for the assay of DAPA and MET in their commercial products during the routine analysis in quality control laboratories.

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