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***Journal of Scientific and Engineering Research*, 2024, 11(4):209-227**

Research Article

ISSN: 2394-2630
CODEN(USA): JSERBR

Development and validation of stability indicating assay methods for estimation of anti-diabetic drugs

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Abstract The goal of the current research was to assess how DAPA and MET degraded under various stress scenarios. According to ICH criteria, the stability indicating HPTLC technique was designed and verified. It was discovered that the procedure was accurate, exact, linear, and specific. The DAPA and MET tablet dose form may be routinely assessed using this approach. Stress tests revealed that DAPA degraded considerably under settings that were acidic, alkaline, oxidative, photolytic, and dry heat degrading. It was discovered that MET was stable under oxidative, photolytic, and dry heat degradation conditions but severely degraded under acidic and alkaline degradation conditions. The approach is able to separate degradation products from DAPA and MET in tablet dosage form as well. Stress testing of DAPA and MET in tablet dosage form also supports the same.

Keywords DAPA, MET, HPTLC, ICH, Stability

Introduction

Diabetes is a group of metabolic disorders in which, blood sugar levels are high over a prolonged period. As per the latest 2016 data from the World Health Organization (WHO), globally, an estimated 422 million adults are suffering with diabetes mellitus. Until recently, India had more number of diabetics compared to any other country in the world, according to the International Diabetes Foundation. But now China has surpassed in the top spot. Diabetes presently affects more than 62 million Indians, which is more than 7.1% of the total adult population. The average age of onset is 42.5 years. Every year, nearly 1 million Indians die due to diabetes.

The WHO estimates that diabetes resulted in 1.5 million deaths in 2012, making it the 8th leading cause of death. However, another 2.2 million deaths worldwide were attributable to high blood glucose and the increased risks of associated complications (e.g. heart disease, stroke, kidney failure), which often result in premature death and are often listed as the underlying cause of death certificates rather than diabetes. So, focusing on diabetesis very important.

Diabetes is an endocrine disorders characterized by

- Deranged secretion of insulin and/or glucagon with extensive disturbance of carbohydrate, protein and lipid metabolism
- Thickening of capillary basement membranes throughout the body and
- Long term complications involving kidney (renal failure), eye (cataract), circulation (cardiomyopathy) and peripheral nervous system (neuropathy)

Maturity-onset or type-II diabetes or non-insulin-dependent diabetes mellitus(NIDDM)

It is more common than Type-I diabetes. It is usually mild; ketoacidosis is rare and is associated with obesity in over two-third of the cases. The β-cells respond normally and plasma insulin levels are normal or raised. Majority of diabetic population (80-90%) suffer from this type of diabetes mellitus [1-5].

Diabetes Insipidus

Diabetes insipidus (DI) is a rare disease that causes frequent urination. A person with DI may feel the need to drink large amount of water and is likely to urinate frequently, even at night, which can disrupt sleep and on occasion, cause bedwetting. Because of the excretion of abnormally large volume of dilute urine, people with DI may quickly become dehydrated if they do not drink enough water. Milder forms of DI can be managed by drinking enough water, usually between 2-2.5 liters a day.

High Performance Thin-Layer Chromatography(HPTLC) [6-14]

High Performance Thin Layer Chromatography (HPTLC) is a very well-known separation method. It shows many advantages in comparison to other separation techniques. The basic method of thin layer chromatography is enhanced in number of ways to automate the different steps, for better resolution and for more accurate quantitative measurements. HPTLC is one of the most simple separation techniques available today. It can be considered as a time saving machine that can speed work and allows doing many things at a time which is not possible with other analytical techniques. HPTLC layer is homogeneous and thin, resulting in shorter analysis time, improved resolution and is compatible for in situ quantization.

In HPTLC method, the stationary phase (fixed phase) is finely divided solid that has been coated with thin layer of inert supporting material. Silica gel is the most commonly used stationary phase used for adsorption TLC and modified silica such as octadecylsilyl (ODS) for reversed phase chromatography. The selection of mobile phase depends on nature of the solute and stationary phase. Optimum mixture of solvents is used as a mobile phase. HPLC has better separation efficiency compared to HPTLC. However, HPTLC is capable of producing fast qualitative results and high-resolution separation with precision and accuracy revealing those of HPLC and GC. Today HPTLC is one of various analytical techniques considered as a reliable method for quantitative analysis of micro, nano and even pictogram level, in complex formulations as well. HPTLC is off-line process in which various stages are carried out independently. The advantage of this arrangement using an open, disposable layer compared with an on-line column process such as HPLC has much higher throughput (analysis time) and lower cost per analysis than HPLC. The ability to process standard and samples simultaneously on a single plate under the similar conditions makes HPTLC a good choice for analyst. It has led to statistical improvement in data handling with better analytical accuracy and precision [12-17].

Material & Method

Preliminary Study of DAPA AND MET

Characterization of drug substances was done by determining melting point, UV spectrum and comparing IR spectrum with reference spectrum.

Determination of melting point

Melting points of DAPA and MET were determined by capillary method using Thiele's tube apparatus and compared with reference melting point.

Solubility analysis

Solubility analysis of DAPA and MET were carried out at 25°C temperature in water, methanol and ethanol.

Determination of UV spectra

UV spectra of DAPA (10 µg/ml) and MET (10 µg/ml) in methanol was recorded against methanol as a blank.

Determination of IR spectra

Infrared spectra of DAPA and MET were taken using FTIR-ATR spectrometer.

Preparation of Standard Solutions

Stock solution of standard DAPA in methanol

Accurately weighed 10 mg DAPA was transferred in to a 10 ml volumetric flask, dissolved in 5 ml of methanol and diluted up to the mark to get stock solution having strength of 1000 µg/ml.

Stock solution of standard MET in methanol

Accurately weighed 50 mg MET was transferred in to a 10 ml volumetric flask, dissolved in 5 ml of methanol and diluted up to the mark with the same to give stock solution having strength of 5000 µg/ml.

Working standard solution of DAPA and MET in methanol

Aliquot 1 ml of DAPA standard stock solution and 5 ml of MET standard stock solution was transferred to 10 ml volumetric flask and dilute up to mark with methanol to get solution of DAPA (100 µg/ml) and MET (2500 µg/ml). 1 ml from this solution is further diluted to 10 ml methanol to get working standard solution of DAPA (10 µg/ml) and MET (250 µg/ml)

Preparation of 1 N hydrochloric Acid

Concentrated Hydrochloric acid (8.5 ml) was transferred into a 100 ml of volumetric flask and diluted up to the mark with double distilled water.

Preparation of 1 N sodium hydroxide

Accurately weighed 4 gm of sodium hydroxide was transferred into 100 ml volumetric flask, dissolved in 50 ml of double distilled water and diluted up to the mark with double distilled water.

Preparation of 3% H2O2

Hydrogen peroxide (6%, 5 ml) was transferred into a 10 ml of volumetric flask and diluted up to the mark with methanol.

Forced Degradation Study

Forced degradation of DAPA and MET were carried out under acidic and alkaline hydrolytic, oxidative, photolytic and dry heat stress conditions. The results of forced degradation study of DAPA and MET are shown in section of result and discussion.

Preparation of sample solutions for forced degradation study of DAPA and MET

DAPA and MET was exposed to acidic (1 N HCl), alkaline (1 N NaOH), oxidative (3% H_2O_2), photolytic (direct sun light for 6 hrs) and dry heat (50°C) degradation conditions forforced degradation study.

Acid hydrolysis

To perform acid degradation study, appropriate aliquots (0.5 ml) of stock solution (1000 µg/ml) of DAPA and (2 ml) of stock solution (5000 µg/ml) of MET were taken in 10 ml volumetric flask and 5 ml of 1 N HCl was added. This solution was kept in a water bath at 60°C for 1 hr and allowed to cool to room temperature. The solution was diluted up to the mark with methanol to get solution of 50 µg/ml DAPA and 1000 µg/ml MET. Further, aliquot of 2 ml was transferred into 10 ml volumetric flask, neutralized with 1N NaOH and diluted up to mark with methanol to get solution of 10 µg/ml DAPA and 200 µg/ml MET. Individual solutions of DAPA and MET were treated similarly.

Alkaline hydrolysis

To perform alkali degradation study, appropriate aliquots (0.5 ml) of stock solution (1000 µg/ml) of DAPA and (2 ml) of stock solution (5000 µg/ml) of MET were taken in 10 ml volumetric flask and 5 ml of 1 N NaOH was added. This solution was kept in a water bath at 60°C for 1 hr and allowed to cool to room temperature. The

solution was diluted up to the mark with methanol to get solution of 50 µg/ml DAPA and 1000 µg/ml MET. Further, aliquot of 2 ml was transferred into 10 ml volumetric flask, neutralized with 1N HCl and diluted up to mark with methanol to get solution of 10 µg/ml DAPA and 200 µg/ml MET. Individual solutions of DAPA and MET were treated similarly.

Oxidative stress degradation

To perform oxidative stress degradation, appropriate aliquots (0.5 ml) of stock solution (1000 µg/ml) of DAPA and (2 ml) of stock solution (5000 µg/ml) of MET were taken in 10 ml volumetric flask and 5 ml of 3% H_2O_2 was added. This solution was kept in a water bath at 60°C for 2 hrs and allowed to cool to room temperature. Then it was diluted up to the mark with methanol to get solution of 50 µg/ml DAPA and 1000 µg/ml MET. Further, aliquot of 2 ml was transferred into 10 ml volumetric flask and diluted up to mark with methanol to get solution of 10 µg/ml DAPA and 200 µg/ml MET. Individual solutions of DAPA and MET were treated similarly

Photo degradation

Analytically pure sample of DAPA and MET were exposed to sunlight for 6 hrs. Then 10 mg of DAPA and 50 mg of MET were weighed, transferred to individual 10 ml volumetric flasks and both were dissolved in 5 ml of methanol. Volume was made up to the mark with the methanol. Aliquot from these solutions (0.5 ml) of solution (1000 µg/ml) of DAPA and (2 ml) of solution (5000 µg/ml) of MET was taken in 10 ml volumetric flask. It was diluted with methanol to obtain 50 µg/ml DAPA and 1000 µg/ml MET. Further, aliquot of 2 ml was transferred into 10 ml volumetric flask and diluted up to mark with methanol to get solution of 10 µg/ml DAPA and 200 µg/ml MET. Individual solutions of DAPA and MET were treated similarly.

Dry heat degradation

For dry heat degradation study, analytically pure samples of DAPA and MET were exposed in oven at 50°C for 2 hrs. Then 10 mg of DAPA and 50 mg of MET were weighed, transferred to individual 10 ml volumetric flasks and both were dissolved in 5 ml of methanol. Volume was made up to the mark with the methanol. Aliquot from this solutions, (0.5 ml) of solution (1000 µg/ml) of DAPA and (2 ml) of solution (5000 µg/ml) of MET were taken in 10 ml volumetric flask. It was diluted with methanol to obtain 50 µg/ml DAPA and 1000 µg/ml MET. Further, aliquot of 2 ml was transferred into 10 ml volumetric flask and diluted up to mark with methanol to get solution of 10 µg/ml DAPA and 200 µg/ml MET. Individual solutions of DAPA and MET were treated similarly. Degradation conditions for forced degradation study of DAPA and MET are summarizedin Table.

Table 1: Degradation conditions for DAPA and MET

Sr No.	Stress type	Stress condition		
		Strength	Heating time	Temperature
1	Acid hydrolysis	1 N HCl	1 hr	60°C
2	Alkaline hydrolysis	1 N NaOH	1 hr	60°C
3	Oxidative	3% H_2O_2	2 hrs	60°C
4	Photolytic	Sunlight	Exposure time 6 hrs	-
5	Dry heat	In oven	2 hrs	50°C

Development of Stability Indicating HPTLC Method

Stability indicating HPTLC method for determination of DAPA and MET was developed by proper selection of mobile phase and wavelength for detection. All the results are mentioned in section of result and discussion.

Optimization of mobile phase

Pre coated silica gel aluminium plate $60F_{254}$ was prewashed with methanol and activated in oven at 50°C for 10 mins. The standard stock solutions of DAPA and MET and their degraded drug solution were spotted separately

on pre coated silica gel aluminium plate by using glass capillary tube and allowed it to dry for few minutes. Different mobile phases (Table) (10 ml) were taken in developing chamber and allowed it to saturate for 15 mins. After saturation, the spotted plate was developed in mobile phase about ¾ height of the plate. The plate was removed and allowed it to dry. Spots were observed in UV cabinet lamp for tailing, shape, separation etc.

Selection of Wavelength of Detection

The standard solutions of DAPA (10µg/ml) and MET (10µg/ml) were scanned in the range of 200-400 nm against methanol as blank in UV visible spectrophotometer. UV overlay spectra of DAPA and MET showed that these two drugs absorbs appreciably at 220 nm.

Optimized Chromatographic Conditions

Separation was performed on 10 × 10 cm aluminium backed plates precoated with 250 µm layer of silica gel 60F254 (E. Merk, Darmstsdt, Germany). Before performing separation, the TLC plates were per-washed with methanol and dried in oven at 50°C for 10 mins. Samples were spotted on TLC plate, 15 mm from the bottom edge using Linomat V semi-automatic spotter and analysed using parameters described in Table.

Table 2: Optimized chromatographic conditions used for HPTLC Method

Parameter	Chromatographic conditions
Stationary phase	Pre-coated silica gel G60 – F aluminium sheet (E. Merck, 254 Germany) (100×100 mm, thickness layer 0.2 mm)
Mobile phase	Methanol: ethyl acetate: ammonium acetate (6: 4: 0.1 v/v/v)
Chamber saturation time	15 min at room temperature (25 ± 2°C)
Distance run	8.0 cm
Detection	220 nm
Measurement mode	Absorbance
Source lamp	Deuterium
Slit dimension	6 mm × 0.45 mm
Syringe capacity	100 µL
Band width	6 mm
Distance from the plate edge	15 mm
Distance from the bottom of the plate	15 M

Solution stability

Freshly prepared working standard solution of DAPA (10 µg/ml) and MET (250 µg/ml) was stored in refrigerator at 5 ± 2°C. This solution was analysed immediately and after 24 hrs duration using optimized chromatographic conditions (Table 6.2). The peak area of the DAPA and MET obtained at 0 hr and after 24 hrs were compared.

Preparation of calibration curve

For the calibration curve, from the mixed working standard solution of DAPA (10 µg/ml) and MET (250 µg/ml) aliquots of 2, 4, 6, 8 and 10 µl were spotted on a TLC plate and analysed as per optimized chromatographic conditions (Table 6.2). Calibration curve was constructed by plotting peak area of DAPA and MET against corresponding DAPA (20- 100 ng/spot) and MET concentration (500-2500 ng/spot).

Analysis of forced degraded samples

From each forced degradation conditions mentioned in Table 6.1, 8 µl of degraded sample solutions (10 µg/ml DAPA and 200 µg/ml MET, mixed and individual) were spotted on TLC plate to get 80 ng/spot of DAPA and

1600 ng/spot of MET. The plate was developed in twin trough chamber, dried and analysed according to optimized chromatographic conditions (Table).
The area of bands of DAPA and MET were measured, compared with that of the standard and percentage degradation of the drug was calculated from the calibration curve.

Determination of DAPA and MET in Tablet Dosage Form
The developed HPTLC method was applied for determination of DAPA and MET in tablet dosage form. The results are shown in section of result and discussion.

Procedure for determination of DAPA and MET
The quantity of tablet powder equivalent to 10 mg of DAPA and 500 mg of MET was transferred to 100 ml volumetric flasks, dissolved in 50 ml of methanol and diluted up to the mark with the same to get solution containing 100 µg/ml of DAPA and 5000 µg/ml of MET. This solution was filtered through whatman filter paper No. 41 and from filtrate, 1 ml was diluted to 10 ml (10 µg/ml of DAPA and 500 µg/ml of MET). Further 4 ml was diluted to 10 ml to get solution of 4 µg/ml of DAPA and 200 µg/ml of MET. The procedure was repeated three times. From all three resulting solutions, 10 µl solution was applied on same TLC plate at three different positions. Plate was developed and analysed as per the optimized chromatographic conditions (Table). The concentration was calculated using equation of straight line.

Procedure for forced degradation study of DAPA and MET in tablet dosage form
The DAPA and MET in tablet dosage form was exposed to forced degradation conditions as described in Table 6.1. The final solution of forced degradation samples were having 4 µg/ml of DAPA and 200 µg/ml of MET. From all degradation conditions, 10 µl solution was applied on TLC plate to get 40 ng/spot of DAPA and 2000 ng/spot of MET. Plate was developed and analysed as per the optimized chromatographic conditions (Table). The concentration was calculated using equation of straight line and percentage degradation was calculated.

Results and Discussion
Preliminary Study of DAPA and MET
Characterization of DAPA and MET was done by determining solubility, melting point, UV spectrum and comparing IR spectrum with reference spectrum.

Solubility analysis
Solubility analysis of DAPA and MET was carried out at 25°C in different solvents. DAPA was found to be slightly soluble in water and freely soluble in ethanol and methanol. MET was found to be freely soluble in water and methanol and slightly soluble in ethanol. The results reveals that DAPA and MET complies in solubility analysis.

Table 3: Solubility analysis of DAPA

Solvent used	Solubility testing	Solubility analysis	Inference
Water	1 part in 200 ml	Slightly soluble	Complies
Methanol	1 part in 10 ml	Freely soluble	Complies
Ethanol	1 part in 10 ml	Freely soluble	Complies

1 part = 1 gm solute

Table 4: Solubility analysis of MET

Solvent used	Solubility testing	Solubility analysis	Inference
Water	1 part in 10 ml	Freely soluble	Complies
Methanol	1 part in 10 ml	Freely soluble	Complies
Ethanol	1 part in 100 ml	Slightly soluble	Complies

1 part = 1 gm solute

Melting point

Melting points of DAPA and MET were found to be 75-80°C and 220-225°C respectively, as shown in Table. Observed values were in good agreement with the reported values.

Table 5: Melting point determination of DAPA and MET

Drug	Standard M.P.	Observed M.P.
DAPA	74-78°C	75-80°C
MET	222-226°C	220-225°C

UV determination

UV spectra of DAPA (10 µg/ml) and MET (10 µg/ml) in methanol are shown in Fig. λmax of the DAPA was found to be 224 nm which match to the reported λ max, 224 nm. λmax of the MET was found to be 233 nm which is very close to the reported λ max, 231 nm.

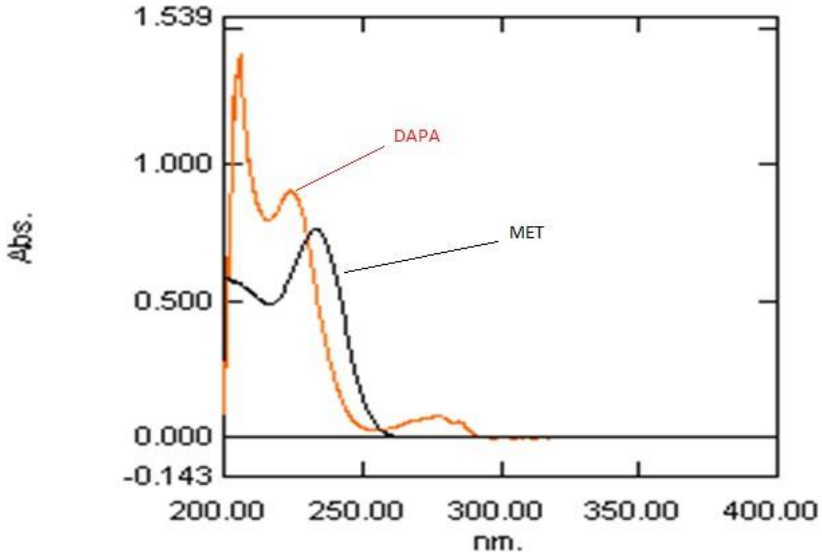

Figure 6: UV Spectra of DAPA (10 µg/ml) and MET (10 µg/ml) in methanol

IR determination

Reference IR spectrum (from literature) of DAPA and sample IR spectrum of DAPA are shown in Figs respectively. The finger print region of the recorded FT-IR spectrum of the drug sample was compared with that of reference spectrum of DAPA. Finger print region of both the spectra was found to be overlapping which confirmed the identity of the obtained sample of drug. The detailed analysis of IR spectrum of DAPA is described inTable.

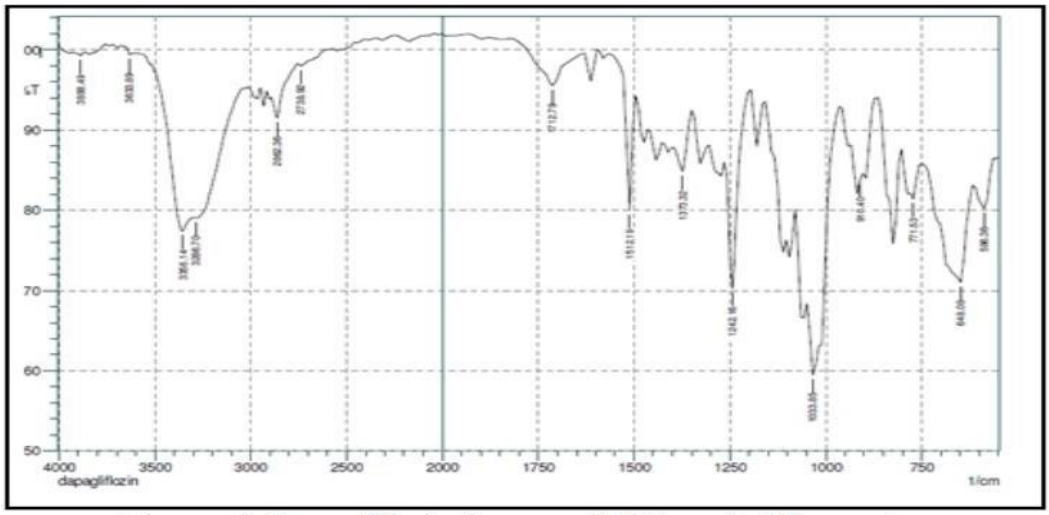

Figure 7: Reference IR spectrum of DAPA

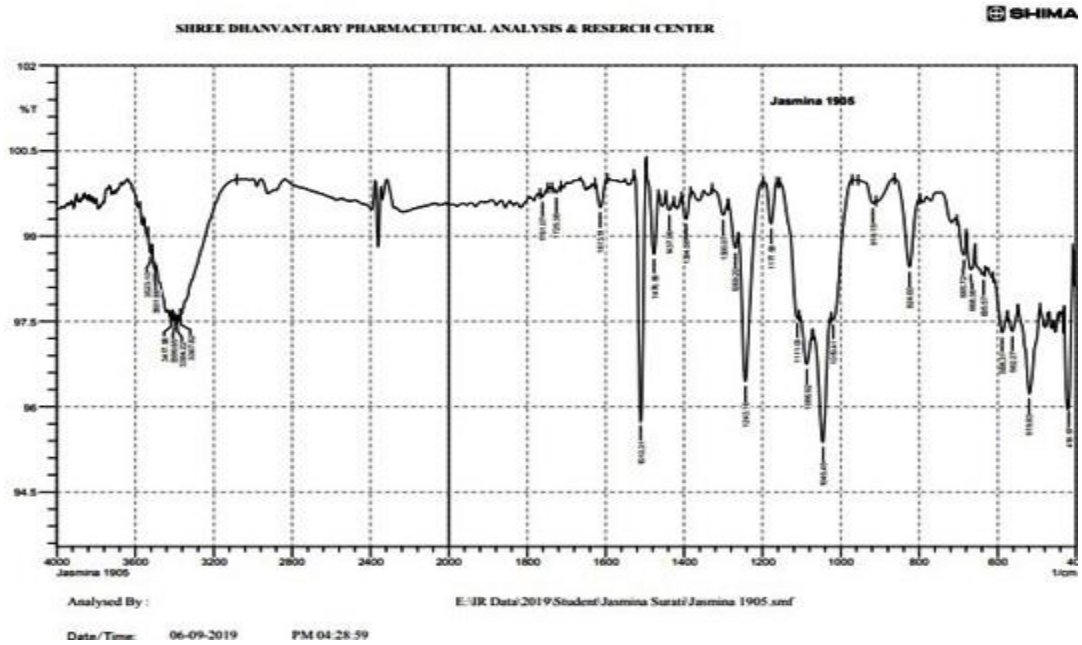

Figure 8: Observed IR spectrum of DAPA

Table 6: Analysis of IR spectrum of DAPA

Sr No	Wave number (cm^{-1}) Reference spectrum	Wave number (cm^{-1}) Sample spectrum	Interpretation
1	3266, 3356	3367-3523 strong broad	O-H stretching
2	1242	1243	C-O stretching
3	1512	1510	C=C stretching
4	650 to 850, 1440, 1500 near	650 to 850, 1400, 1500 near	Indicate presence of Benzene ring
5	648	824	C-Cl stretching

Reference IR spectrum (from literature) of MET and sample IR spectrum of MET are shown in Fig. respectively. The finger print region of the recorded FT-IR spectrum of the drug sample was compared with that of reference spectrum of MET. Finger print region of both the spectra was found to be overlapping which confirmed the identity of the obtained sample of drug. The detailed analysis of IR spectrum of MET is described in Table.

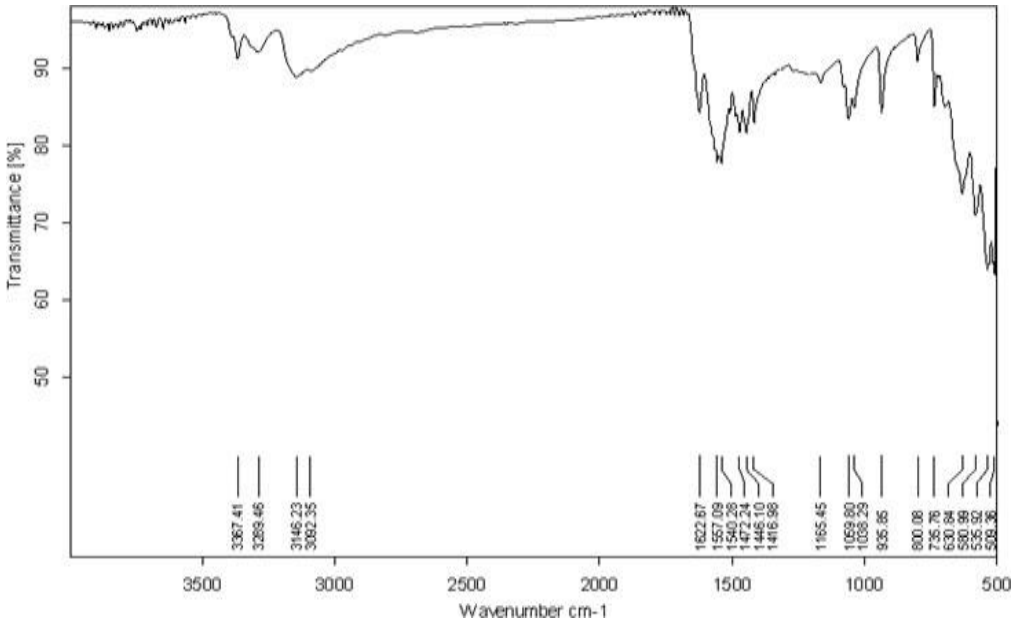

Figure 9: Observed IR spectrum of MET

Table 7: Analysis of IR spectrum of MET

Sr No	Wave number (cm^{-1}) Reference spectrum	Wave number (cm^{-1}) Sample spectrum	Interpretation
1	1700-1600	1622	C = N stretching
2	3500-3100	3367, 3289, 3146	N-H stretching
3	1650-1550	1567, 1540	N-H bending
4	1350-1000	1165	C-N stretching
5	-	530	C-N-C deformation

Development and Validation of Stability Indicating HPTLC Method

Mobile Phase Optimization

Drug solutions and degraded solutions from different stress conditions were spotted on the TLC plates separately and were run in different solvent systems (Table). The mobile phase Methanol: ethyl acetate: ammonium acetate (6: 4: 0.1 v/v/v) gave good resolution and compact spot with Rf values of 0.31 and 0.79 for MET and DAPA, respectively and it can separate all the degradation products of DAPA and MET in different stress conditions. Densitogram for DAPA std., MET std. and mixture of DAPA and MET are shown in Fig. respectively.

Table 8: Optimization of mobile phase

Sr. No.	Mobile Phase Composition	Result
1.	Methanol	Light spots observed for DAPA and no spot seen for MET
2.	Methanol : Toluene (2:8 v/v)	DAPA Spot observed and MET spot have tailing
3.	Methanol : Chloroform (2:8 v/v)	No separation of the both drug and their degradation products.
4.	Methanol : Water(2:8 v/v)	Clear spots seen for DAPA and no spot observed for MET.
5.	Toluene: water (3:7 v/v)	No separation of drugs
6.	Toluene: ethanol (2:8 v/v)	No separation of drugs
7.	Acetone : Methanol (8:2v/v)	Spots are seen near the solvent front for DAPA while tailing is seen for MET and degradation product of both drug were not separated.
8.	Methanol: ethyl acetate: ammonium acetate (5: 5: 0.1 v/v/v)	Good separation with clear spots but degradation product in acid and base were not separated.
9.	Methanol: ethyl acetate: ammonium acetate (5.5: 4.5: 0.1 v/v/v)	Good separation with clear spots but tailing is observed and poor separation of degradation product in DAPA in acidic condition.
10	Methanol: ethyl acetate: ammonium acetate (6: 4: 0.1 v/v/v)	Good separation with clear spots for DAPA and MET. No tailing is observed and good separation of degradation product in all the conditions.

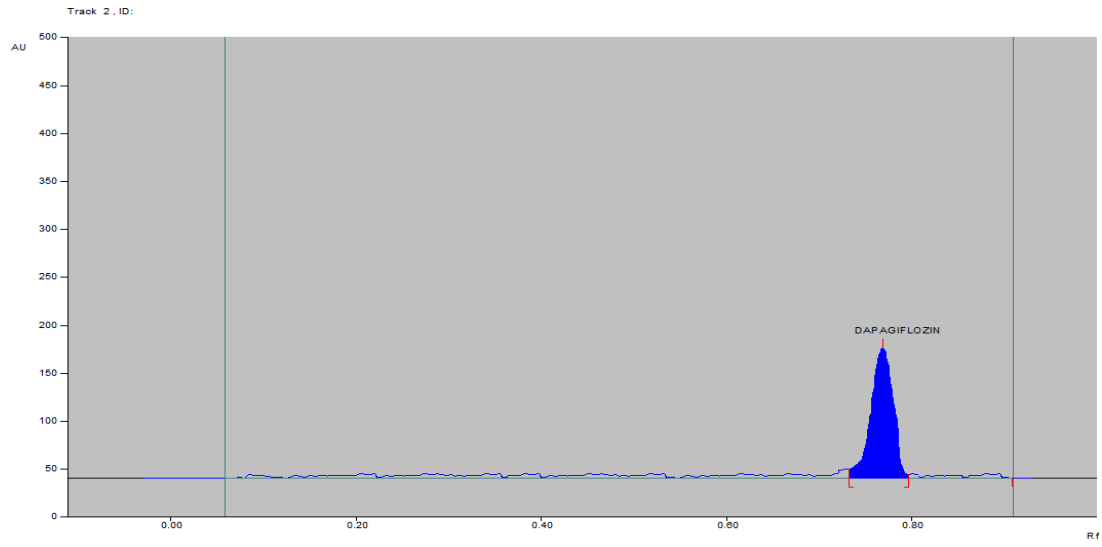

Figure 10: Densitogram of DAPA standard

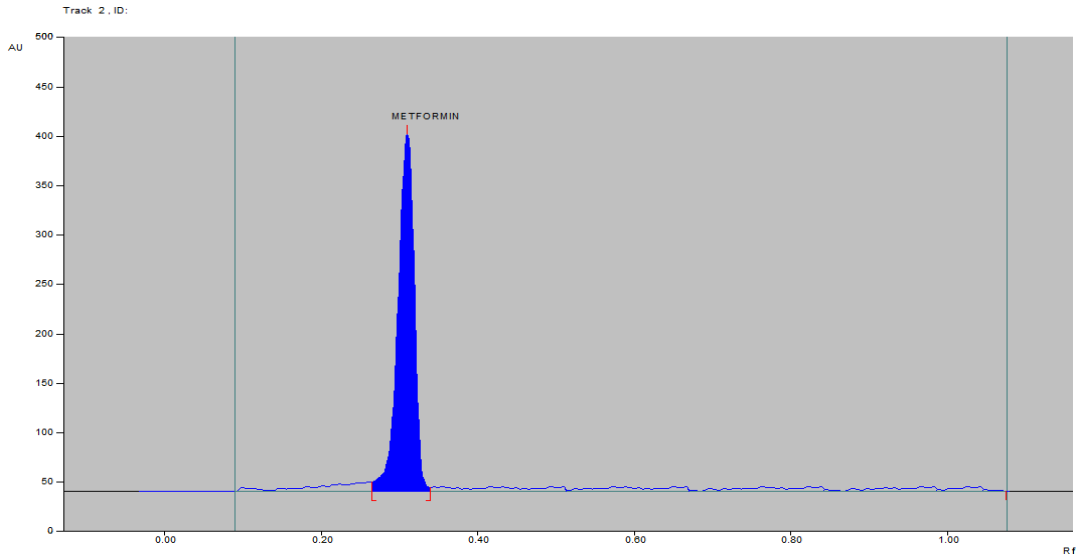

Figure 11: Densitogram of MET standard

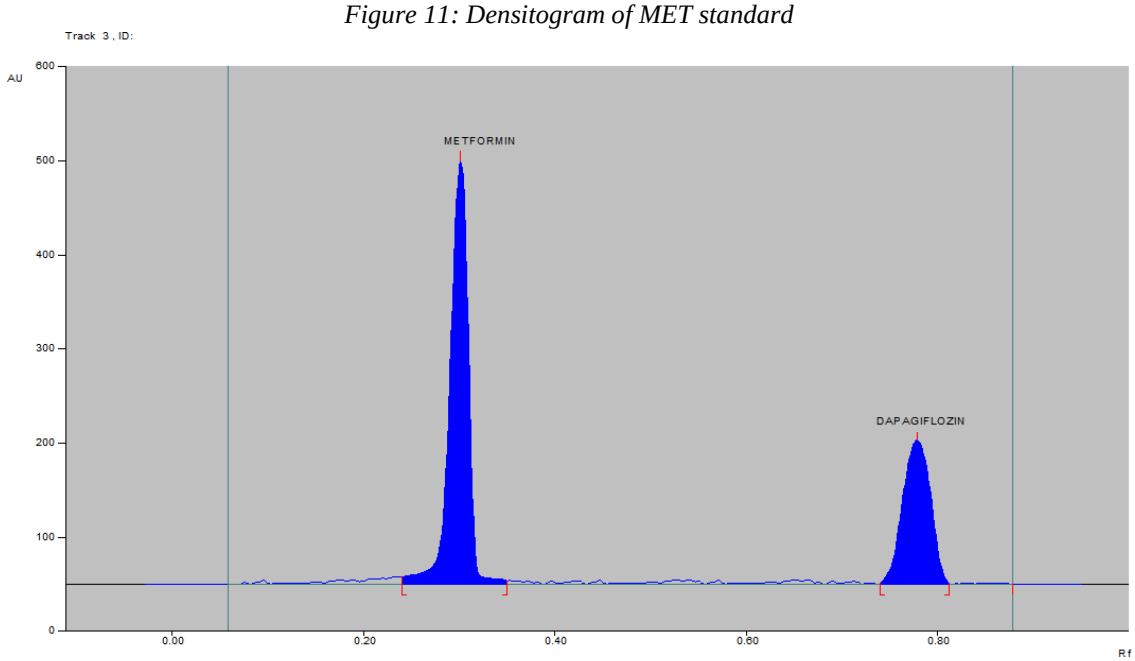

Figure 12: Densitogram of mixture of DAPA and MET standard

Selection of Wavelength of Detection

UV spectrum obtained by scanning solution of DAPA and MET between 200 nm to 400 nm. 220 nm was selected as wavelength for determination of DAPA and MET.

Solution stability

The initial area of DAPA (60 ng/spot) and MET (1500 ng/spot) band from the freshly prepared working standard solution was found to be 1953.33 ± 14.04 and 4939.00 ± 19.97 (n = 3), respectively. The area of DAPA (60 ng/spot) and MET (1500 ng/spot) in same solution analysed after 24 hrs was found to be 1939.00 ± 16.82 and 4922.67 ± 31.09 (n = 3), respectively. The difference between initial area and area after 24 hrs was found to be negligible. Hence the working standard solution of DAPA and MET need to be stored in refrigerator at temperature(5 ± 2°C).

Calibration curve

Calibration curve was prepared in the range of 20 - 100 ng/spot (n = 5) for DAPA and 500 - 2500 ng/spot (n = 5) for MET. They were found to be linear in the above concentration range. Chromatogram, calibration curve of DAPA and calibration curve of MET are shown in Fig. respectively. The optimized chromatographic conditions are shown in Table.

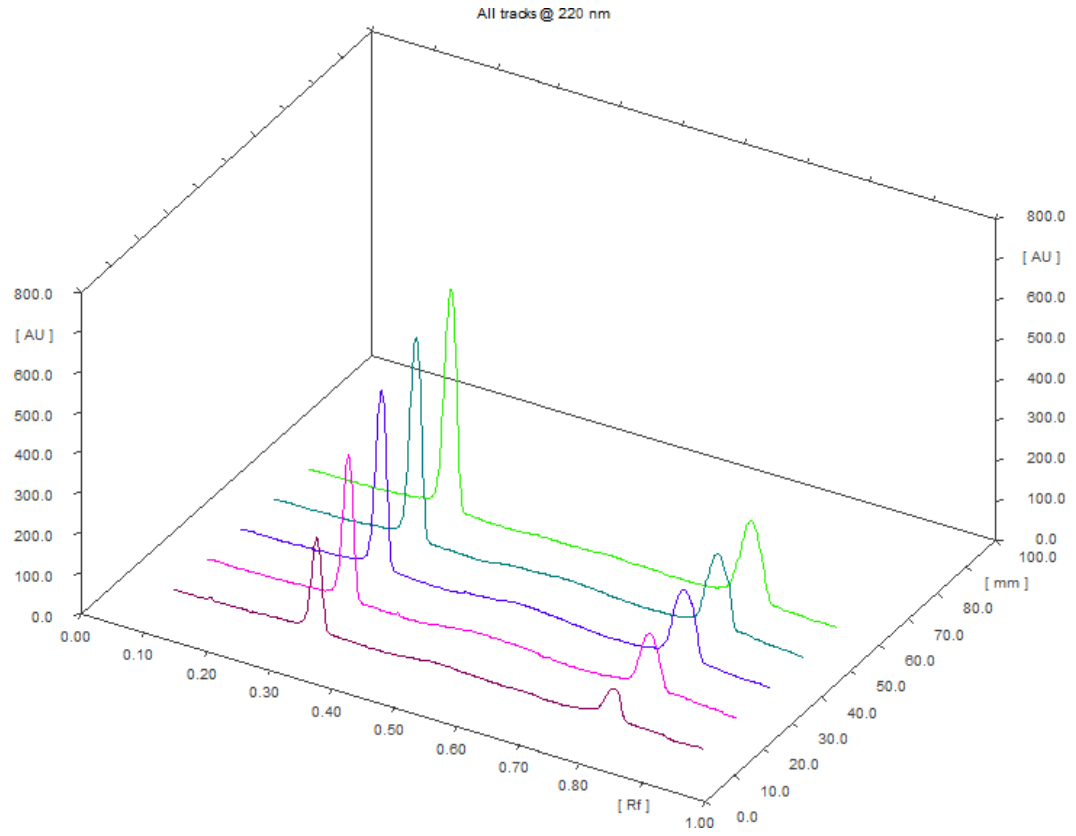

Figure 12: 3D Chromatogram of different concentrations of DAPA (20 - 100ng/spot) and MET (500 - 2500 ng/spot)

Table 9: Optimized chromatographic conditions

Parameter	Chromatographic conditions
Stationary phase	Pre-coated silica gel G60 – F aluminium sheet (E. 254 Merck, Germany) (100×100 mm, thickness layer 0.2mm)
Mobile phase	Methanol: ethyl acetate: ammonium acetate (6: 4: 0.1 v/v/v)
Chamber saturation time	15 min at room temperature (25 ± 2°C)
Distance run	8.0 cm
Detection	220 nm
Measurement mode	Absorbance
Source lamp	Deuterium
Slit dimension	6 mm × 0.45 mm
Syringe capacity	100 µL
Band width	6 mm
Distance from the plate edge	15 mm
Distance from the bottom of the plate	15 mm

Forced Degradation Study

Acidic hydrolysis

Densitogram of acid (1.0 N HCl) treated DAPA, MET and mixture DAPA and MET are shown in Fig. respectively. Data of acidic degradation of DAPA and MET are shown in Table 6.11. It is seen that after heating drug solution with 1.0 N hydrochloric acid at 60°C for 1 hr, significant degradation was observed for both DAPA and MET. One additional peaks at Rf values 0.60, and Rf values 0.45 were observed for DAPA and MET respectively, in the chromatogram of acid treated sample as compared to chromatogram of standard sample. The percentage degradation was found to be 34.98 % and 25.02 % for DAPA and MET, respectively. It proves that DAPA and MET both are degraded in acidic degradation condition. The method was able to separate the drugs from its acidic degradation products.

Table 10: Data of acid degradation of DAPA and MET in 1.0 N HCl

Peak	Rf	Description
1	0.31	MET
2	0.45	Degradation Product 1 of MET
3	0.60	Degradation Product 1 of DAPA
4	0.79	DAPA

Alkaline hydrolysis

Densitogram of alkaline (1.0 N NaOH) treated DAPA, MET and mixture DAPA and MET are shown in Fig respectively. Data of alkaline degradation of DAPA and MET are shown in Table. It is seen that after heating drug solution with 1.0 N sodium hydroxide at 60°C for 1 hr, significant degradation was observed for both DAPA and MET. One additional peaks at Rf values 0.69, and Rf values 0.49 were observed for DAPA and MET respectively, in the chromatogram of alkali treated sample as compared to chromatogram of standard sample. The percentage degradation was found to be 29.12% and 22.87% for DAPA and MET, respectively. It proves that DAPA and MET are degraded in alkaline degradation condition. The method was able to separate the drugs from its alkalinedegradation products.

Table 11: Data of alkali degradation of DAPA and MET in 1.0 N NaOH

Peak	Rf	Description
1	0.31	MET
2	0.49	Degradation Product 2 of MET
3	0.69	Degradation Product 2 of DAPA
4	0.79	DAPA

Oxidative degradation

Densitogram of H_2O_2 (3%) treated DAPA, MET and mixture DAPA and MET are shown in Fig. respectively. Data of oxidative degradation of DAPA and MET are shown in Table 6.13. It is seen that after heating drug solution with 3% H_2O_2 at 60°C for 2 hrs, significant degradation was observed for DAPA, but no degradation was observed for MET. One additional peaks at Rf values 0.58 for DAPA was observed, in the chromatogram of H_2O_2 treated sample as compared to chromatogram of standard sample. The percentage degradation was found to be 25.29% and 2.50% for DAPA and MET, respectively. It proves that DAPA is degraded in oxidative degradation condition, while MET is stable in oxidative degradation condition. The method was able to separate DAPA from its oxidative degradation products.

Table 12: Data of oxidative degradation of DAPA and MET in 3% H_2O_2

Peak	Rf	Description
1	0.31	MET
2	0.58	Degradation Product 3 of DAPA
3	0.79	DAPA

Photolytic degradation

Densitogram of sunlight treated DAPA, MET and mixture DAPA and MET are shown in Fig. respectively. Data of photolytic degradation of DAPA and MET are shown in Table 6.14. It is seen that after exposing drug to direct sunlight for 6 hrs, significant degradation was observed for DAPA, but no degradation was observed for MET. One additional peak at Rf value 0.53 for DAPA was observed, in the chromatogram of H_2O_2 treated sample as compared to chromatogram of standard sample. The percentage degradation was found to be 32.55% and 2.22% for DAPA and MET, respectively. It proves that DAPA is degraded, while MET is stable in photolytic degradation condition. The method was able to separate DAPA from its photolytic degradation product.

Table 13: Data of Photolytic degradation of DAPA and MET in sunlight

Peak	Rf	Description
1	0.31	MET
2	0.53	Degradation Product 4 of DAPA
3	0.79	DAPA

Dry heat degradation

Densitogram of dry heat treated DAPA, MET and mixture DAPA and MET are shown in Fig. respectively. Data of dry heat degradation of DAPA and MET are shown in Table. It is seen that after exposing drug in oven at 50°C for 2 hrs, significant degradation was observed for DAPA, but no degradation was observed for MET. Two additional peak at Rf value 0.48 and 0.54 for DAPA was observed, in the chromatogram of dry heat degraded sample as compared to chromatogram of standard sample. The percentage degradation was found to be 38.25 % and 1.74 % for DAPA and MET, respectively. It proves that DAPA is degraded, while MET is stable in dry heat degradationcondition. The method was able to separate DAPA from its dry heat degradation product.

Table 14: Data of dry heat degradation of DAPA and MET in oven at 50°C

Peak	Rf	Description
1	0.31	MET
2	0.50	Degradation Product 5 of DAPA
3	0.58	Degradation Product 6 of DAPA
4	0.79	DAPA

Summary of Forced Degradation Study

The summary of degradation products of DAPA and MET in various degradation conditions is described in Table. The percentage degradation for DAPA in acidic hydrolysis, alkaline hydrolysis, oxidative, photolytic, thermal degradation condition were found to be 34.98 %, 29.12 %, 25.29 %, 32.55% and 38.25% respectively. The percentage degradation for MET in acidic hydrolysis, alkaline hydrolysis, oxidative, photolytic, thermal degradation condition were found to be 25.02 %, 22.87 %, 2.50 %, 2.22 % and 1.74 % respectively. Hence it can be concluded that DAPA is degraded in all degradation conditions. While MET is stable in oxidative, photolytic and dry heat degradation conditions.

Table 15: Summary of degradation products of DAPA and MET in various stress conditions

Force degradation condition	Stress Condition	DAPA			MET		
		Rf value of degradants	Drug remain undergraded (%)	Degradation (%)	Rf value of degradants	Drug remain undergaded (%)	Degradation (%)
Acidic	(1 N HCl / 60°C/1hr)	0.6	65.02	34.98	0.45	74.98	25.02
Alkaline	(1 N NaOH/ 60°C/1hr)	0.69	70.88	29.12	0.49	77.13	22.87
Oxidative	(3% v/v H_2O_2/ 60°C/2 hrs)	0.58	74.71	25.29	-	97.50	2.50
Photo	6 hrs in sunlight	0.53	67.45	32.55	-	97.78	2.22
Thermal	(50°C / 2 hrs)	0.50 0.58	61.75	38.25	-	98.26	1.74

Validation of Developed Method

Linearity and Range

Representative calibration curve of DAPA and MET were obtained by plotting the mean peak area of DAPA against concentration over the range of 20 – 100 ng/spot (n=3) (Fig.) and plotting the mean peak area of MET against concentration over the range of 500 – 2500 ng/spot (n=3) (Fig.). Responses were found to be linear in the above concentration range with correlation coefficients of 0.9985 and 0.9984 for DAPA and MET respectively. The results of linearity are shown in Table. The %RSD for DAPA and MET were found to be in the range of 0.69 - 1.85% and 0.76 - 1.31%, respectively. The average linear regressed equation for the curve of DAPA and MET were found to be y = 33.95x – 112 and y = 3.58x - 242.53, respectively. The areas obtained are directly proportional to the concentration of analyte based on the linearity results. %RSD value less than 2% clearly indicate that the developed method is linear in range of 20 – 100 ng/spot for DAPA and 500 – 2500 ng/spot for MET.

Table 16: Linearity data for DAPA and MET

DAPA			MET		
Concentration (ng/spot)	**Peak area (mean ± S.D.) (n=3)**	**%RSD**	**Concentration (ng/spot)**	**Peak area (mean ± S.D.) (n=3)**	**%RSD**
20	571 ± 10.54	1.85	500	1662 ± 21.73	1.31
40	1240± 11.53	1.42	1000	3284± 26.95	0.82
60	1959± 31.94	1.63	1500	4961± 38.00	0.77
80	2538± 17.62	0.69	2000	6908± 64.63	0.94
100	3318± 24.03	0.72	2500	8788± 66.53	0.76

Specificity

The spots of DAPA and MET in tablet powder were confirmed by comparing its Rf and absorbance-reflectance spectrum with that of standard DAPA and MET. spectra scanned at peak start (s), peak apex (m) and peak end (e) position of individual spots of DAPA and MET was compared, which was showing a high degree of correlation confirmed the purity of the corresponding spots. Absorbance-reflectance spectra of standard DAPA and MET, sample DAPA and MET, overlain spectra of standard and sample DAPA and MET are shown in Fig. Data of Peak Purity for DAPA and MET is shown in Table 6.18**.** From the overlain spectrum of DAPA and MET standard and DAPA and MET sample, identity of the drug was confirmed. The results indicate that the developed method is specificfor DAPA and MET.

Table 17: Data of Peak Purity for DAPA and MET

Drug	**r (s,m)**	**r (m,e)**
DAPA	0.9997	0.9992
MET	0.9999	0.9999

Precision

Repeatability of measurement and Repeatability of sample application

Precision of the instrument was checked by repeated scan of the same spot (6 µl working standard solution of DAPA (10 µg/ml) and MET (250 µg/ml)) seven times without changing the plate position. The data is depicted in Table. The % RSD for measurement of peak area was found to be 0.48 % and 0.44 % for DAPA and MET, respectively. Low value of % RSD clearly ensures precision of the measuring device. The repeatability of sample application was checked by application of working standard solution, 6 µl working standard solution of DAPA (10 µg/ml) and MET (250 µg/ml) seven times on the same plate. The data is depicted in Table. The %RSD for measurement of peak area was found to be 0.71% and 0.67% for DAPA and MET, respectively, which ensures precision of the spotter device.

Table 18: Repeatability data of DAPA and MET

DAPA			MET		
Concentration(ng/spot)	**Repeatabilityof Peak area measurement**	**Repeatabilityof sample applicationPeak area**	**Concentration (ng/spot)**	**Repeatabilityof Peak area measurement**	**Repeatabilityof sample applicationPeak area**
60	1972	1972	1500	4923	4923
60	1963	1979	1500	4930	4902
60	1960	1968	1500	4923	4908
60	1978	1996	1500	4919	4963
60	1964	1953	1500	4924	4927
60	1987	1987	1500	4977	4985
60	1974	1982	1500	4944	4972
Mean	1971.14	1976.71	**Mean**	4934.29	4940.00
SD	9.56	13.97	**SD**	21.83	32.92
% RSD	0.48	0.71	**% RSD**	0.44	0.67

Intermediate precision

Three concentrations viz., 40, 60 and 80 ng/ spot of DAPA and 1000, 1500 and 2000 ng/ spot of MET were analysed on same day at three time intervals for intraday precision and on three consecutive days for interday precision. The data for intraday precision and interday precision for DAPA and MET is presented in Tables respectively. The % RSD for intraday precision was found to be 1.01 - 1.29% and 0.67 - 1.23% for DAPA and MET, respectively. %RSD for interday precision was found to be 1.14 - 1.72% and 0.70 – 1.59% for DAPA and MET, respectively. %RSD value less than 2% clearly indicate that thedeveloped method is precise.

Table 19: Intermediate precision data for DAPA

Concentration (ng/spot)	**Intraday precision**		**Interday precision**	
	Area (Mean ± SD) (n=3)	**% RSD**	**Area (Mean ± SD) (n=3)**	**% RSD**
40	1238.33 ± 15.95	1.29	1263.33 ± 21.73	1.72
60	1943.00 ± 20.00	1.03	1963.33 ± 27.06	1.38
80	2490.33 ± 25.15	1.01	2549.67 ± 29.02	1.14

Table 20: Intermediate precision data for MET

Concentration (ng/spot)	**Intraday precision**		**Interday precision**	
	Area (Mean ± SD) (n=3)	**% RSD**	**Area (Mean ± SD) (n=3)**	**% RSD**
1000	3299.33 ± 40.46	1.23	3270.33± 51.93	1.59
1500	4951.67 ± 33.31	0.67	4949.67 ± 34.43	0.70
2000	6905.33 ± 63.96	0.93	6914.67 ± 72.57	1.05

Accuracy

Accuracy of the method was confirmed by recovery study from tablet powder at three levelsof standard addition. The data shown in Tables indicate that the developed method is accurate. The % recovery was found to be in range of 99.95 – 101.58%and 99.70 – 101.09% for DAPA and MET, respectively.

Table 21: Accuracy data for DAPA

Track	DAPA from tablet powder (ng/spot)	Spiked amount of standard DAPA (ng/spot)	Total amount of DAPA (ng/spot)	Amount recovered (Mean ± SD) (n=3)	Mean % recovery
1	20	-	20	-	-
2	20	16	36	16.04 ± 0.24	100.27
3	20	20	40	19.99 ± 0.32	99.95
4	20	24	44	24.38 ± 0.34	101.58

TABLE 22 Accuracy data for MET

Track	MET from tablet powder (ng/spot)	Spiked amount of standard MET (ng/spot)	Total amount of MET (ng/spot)	Amount recovered (Mean ± SD) (n=3)	Mean % recovery
1	1000	-	100	-	-
2	1000	800	1800	797.57 ± 3.36	99.70
3	1000	1000	2000	1003.50 ± 3.94	100.34
4	1000	1200	2200	1213.08 ± 2.91	101.09

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ for DAPA and MET were calculated from value of Y- intercept and slope of five different calibration curves. The data for LOD and LOQ of DAPA and MET are depicted in Table. The LOD and LOQ for DAPA were found to be 3.12 ng/spot and 9.48 ng/spot, respectively. The LOD and LOQ for MET were found to be 11.47 ng/spot and 34.76 ng/spot, respectively. Such a low value of LOD and LOQ confirms sensitivity of method.

Table 23: Data of LOD and LOQ for DAPA and MET

Parameters	DAPA	MET
Mean Y- intercept ± S.D.(n=5)	106.92 ± 32.40	246.40 ± 12.46
Mean slope ± S.D.(n=5)	34.18 ± 0.38	3.59 ± 0.0022
LOD = 3.3 × (SD/Slope) (ng/spot)	3.12	11.47
LOQ = 10 × (SD/Slope) (ng/spot)	9.48	34.76

The validation of the developed stability indicating HPTLC method for determination of DAPA and MET indicates that the method is specific, linear, precise and accurate. The summary of different validation parameters is presented in Table.

Table 24: Summary of validation parameters for HPTLC method for determinationof DAPA and MET

Parameters	DAPA	MET
Linearity range	20-100 ng/spot	500-2500 ng/spot
Regression line equation	y = 33.953x - 112	y = 3.5755x - 242.53
Correlation co-efficient (R^2)	0.9985	0.9984
Precision (%RSD)		

Repeatability of measurement (n=7)	0.49	0.44
Repeatability of sample application (n=7)	0.71	0.67
Intra-day precision (n=3)	1.01-1.29	0.67-1.23
Inter-day precision (n=3)	1.14 -1.72	0.70-1.59
% Recovery (n=3)	99.95 – 101.58	99.70 – 101.09
Limit of Detection (LOD)(ng/spot)	3.12	11.47
Limit of Quantitation (LOQ) (ng/spot)	9.48	34.76

The stability indicating method is one of the analytical tool that help to evaluate stability of drug substances under influence of various degradation conditions. It also can be used to evaluate possible degradation product in final dosage form. The combination of DAPA and MET is not official in any of the pharmacopoeias, so no official method is available for analysis of DAPA and MET and its degradation products. Present study aimed at evaluating degradation behaviour DAPA and MET in different stress conditions. Stability indicating HPTLC method was developed and validated as per ICH guidelines. The method was found to be specific, linear, precise and accurate. The method can be used for routine assessment of DAPA and MET in tablet dosage form. From stress testing, DAPA was found to be significantly degrading in acidic, alkaline, oxidative, photolytic and dry heat degradation conditions. While, MET was found to be significantly degrading in acidic and alkaline degradation conditions, while stable in oxidative, photolytic and dry heat degradation conditions. Stress testing of DAPA and MET in tablet dosage form also supports the same and the method is able to separate degradation products from DAPA and MET in tablet dosage form as well.

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