

STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF METFORMIN HYDROCHLORIDE AND PIOGLITAZONE HYDROCHLORIDE IN DOSAGE FORM

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Stability-indicating high performance liquid chromatographic (HPLC) method was developed for simultaneous analysis of metformin hydrochloride (MET) and pioglitazone hydrochloride (PIO) in dosage forms. Chromatographic separation was performed on C₈ column (Qualisil BDS 250 mm × 4.6 mm, 5 μm) with a mixture of methanol and water at 45:55 (v/v) containing 0.2% (w/v) n-heptanesulfonic acid (HSA) and 0.2% (v/v) triethyl amine (TEA) as mobile phase. The flow rate was 1 mL/min and eluents were detected at 265 nm. The described method shows linearity between 100–750 μg/mL for MET and 5–30 μg/mL for PIO with respective correlation coefficient (r²) values of 0.9996 and 0.9997. Drugs were subjected to acidic and basic hydrolysis, oxidative, photolytic, neutral and thermal degradations. This method revealed 14 degradation products and among these products D1, D3, D11, D12 and D14 were identified using impurity standards.

Keywords: RP-HPLC, Stability indicating method, Metformin hydrochloride, Pioglitazone hydrochloride

INTRODUCTION

Metformin hydrochloride (MET) is chemically known as 3-(diaminomethylidene)-1,1-dimethylguanidine hydrochloride and is used as an antidiabetic agent (Fig. 1). It is the drug of choice for the treatment of type II diabetes, particularly in overweight, obese people and individuals with normal kidney function. It works by lowering blood sugar and helping the body to use insulin more efficiently.

The chemical name for pioglitazone hydrochloride (PIO) is 5-[4-[2-(5-ethyl-2-pyridinyl)ethoxy]benzyl]-1,3-thiazolidine-2,4-dione hydrochloride (Fig. 2). It is used in the management of type II diabetes mellitus [also known as non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes]. Pharmacological studies indicate that PIO improves sensitivity to insulin in muscle and adipose tissue and inhibits hepatic gluconeogenesis. Sun Pharmaceutical Industries Ltd. markets PIO in combination forms as Pioglit-MF. In combination these are available in 15:500 mg of PIO and MET, respectively.

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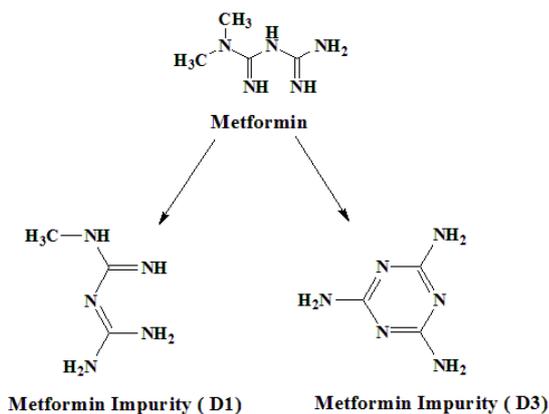


Fig. 1: Structures of MET, impurity-D1 and impurity-D3.

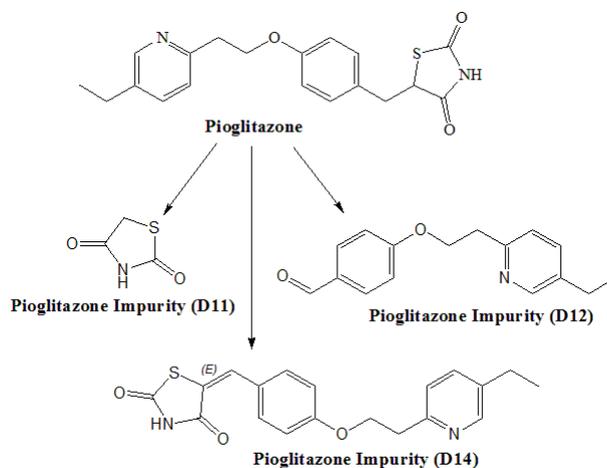


Fig. 2: Structures of PIO, impurity-D11, impurity-D12 and impurity-D14.

Literature reveals that various methods, for instance UV (Sujana *et al.* 2010; Ajithdas and Nancy 2000), high performance liquid chromatography (HPLC) (Jeyabalan and Nyola 2012; Srinivas *et al.* 2012; Lad *et al.* 2003) and ion-pair HPLC (Vasudevan *et al.* 2001) have been reported for individual estimations of MET and PIO, along with other drugs in various dosage forms. PIO is not yet official in any of the pharmacopoeia but MET is official in Indian Pharmacopoeia (1996), British Pharmacopoeia (2002), and United States Pharmacopoeia and The National Formulary (2005). Literature survey reveals that few bio-analytical liquid chromatography-mass spectrometry (LCMS) (Lin *et al.* 2003; Xue *et al.* 2003; Yamashita *et al.* 1996) and stability indicating HPLC (Navaneethan, Karunakaran and Elango 2011) methods were reported for the determination of PIO with other drugs. To date, no stability indicating assay method (SIAM) is available for the simultaneous estimation of MET and PIO in tablet dosage form in pharmaceutical preparations, which promoted the present work. The main objective of this present work is to develop and validate a stability indicating reverse phase HPLC (RP-

HPLC) method for the simultaneous estimation of MET and PIO that could demonstrate all possible impurities in combined dosage forms. Herein we report a stability-indicating RP-HPLC method for simultaneous determination of MET and PIO in the presence of their degradation products in accordance to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines.

EXPERIMENTAL

Chemicals and Reagents

Samples of MET and PIO were obtained as gifts from Hetero Labs Pvt. Ltd. (Hyderabad, Andhra Pradesh, India). Tablet formulation of MET and PIO (Pioglit-MF tablet) were procured from commercial market (Sun Pharmaceutical Industries Ltd., Hyderabad, India). All the solutions were protected from light and were analysed on the day of preparation. Glassware used in each procedure were soaked overnight in a mixture of chromic acid and sulfuric acid rinsed thoroughly with double distilled water and dried in hot air oven. Water and methanol (HPLC grade) were purchased from Merck (Worli, Mumbai).

HPLC Instrumentation and Conditions

HPLC analyses for method development, forced degradation studies and method validation were performed using an Agilent-1200 binary pump plus manual sampler and Agilent photodiode array detector (PDA) (Agilent Technologies India Pvt. Ltd., New Delhi). The output signal was monitored and processed using Ezchrome elite software resident in a Pentium computer (Digital Equipment) from Agilent Technologies India Pvt. Ltd. (New Delhi). Compounds were separated on a 250 mm × 4.6 mm, 5 µm particle, Agilent C₈ column with methanol and water (45:55, v/v) with 0.2% (w/v) n-heptanesulfonic acid (HSA), 0.2% (v/v) triethyl amine (TEA) and pH was adjusted to 3.0 with orthophosphoric acid (OPA) as mobile phase. The injection volume was 20 µL, the mobile phase flow rate was 1.0 mL/min and the detection wavelength was 265 nm.

Forced Degradation Studies

Forced degradation of MET and PIO drug substances was carried out under neutral, acidic, basic, oxidative, thermal and photolytic stress conditions. In stress study, aliquots of stress sample were diluted with mobile phase and achieved a concentration of 100 µg/mL. The pH of stress sample was adjusted to 3–4 and injected in the optimised conditions with appropriate blanks. The samples from acid hydrolysis were neutralised with 0.1N NaOH and the samples from base hydrolysis were neutralised with 1N HCl. Blank solutions for each hydrolysis were prepared at the same time of preparation of stock solutions. The percentage degradation was calculated using response factor based on the peak area.

Preparation of Stock Solution for Stress Studies

An accurately weighted quantity of 10 mg of each drug substance was carefully transferred into a 10 mL volumetric flask, dissolved completely in methanol and the volume was made up to the mark to get 1000 µg/mL. The same procedure was used to prepare stress solutions of acid hydrolysis, base hydrolysis and oxidation respectively with 1N HCl, 0.1N NaOH and 3% H₂O₂. Thermal degradation was carried out for solid state by

the means of heating the samples over a period (0, 3, 6 h) in hot air oven, at 105°C. Photo degradation was carried on sample solutions as in the outlined procedure in the following section. In all stress studies, stress was performed on individual MET and PIO (control) as well as in combination. The results of the individual degradation studies of MET and PIO (control) were compared with degradation profile of combined studies.

Hydrolysis

The stock solutions of 1000 µg/mL were prepared in 0.1N NaOH (basic), 1N HCl (acidic) and methanol (neutral) at room temperature. Samples (1 mL) were withdrawn at different time points (0, 3, 12, 48 h) and made to 10 mL with mobile phase (100 µg/mL). The samples from acid hydrolysis were neutralised with 0.1N NaOH and the samples from base hydrolysis were neutralised with 1N HCl. Blank solutions for each hydrolysis were prepared at the same time of preparation of stock solutions.

Oxidation

The study was carried for period of 10 days. Every day, 1 mL of sample was withdrawn and transferred into 10 mL volumetric flask and made to 10 mL with mobile phase and injected into the optimised conditions at various time intervals against a blank.

Thermal Degradation

Solid state stability studies were performed using preheated sample as thin layer in the petri dish at 105°C. At various time intervals (0, 3, 6 h), the heated samples (10 mg) were weighed, suitably dissolved and diluted with mobile phase to get a concentration of 100 µg/mL and injected into the HPLC system.

Photo Degradation

Photo degradation studies were conducted by exposing the solution sample in sunlight for a total period of 80 h. After degradation, samples taken at different time intervals (0, 12, 24, 48, 70 h) were suitably diluted in mobile phase to a concentration of 100 µg/mL and injected into the HPLC system.

Preparation of Sample Solution for Assay

Twenty tablets of marketed formulation Pioglit-MF containing PIO (15 mg) and MET (500 mg) were weighed and finely powdered. Tablet powder equivalent to 100 mg PIO with relevant quantities of MET was weighed and transferred to a 100 mL volumetric flask, extracted for 30 min with methanol and the volume was made up to 100 mL with diluent. A 0.1 mL aliquot of the above solution was taken in a 10 mL volumetric flask and then made up to 10 mL with mobile phase. The final solution was filtered through 0.45 micron syringe filter and analysed. The results of the assay were shown in Table 1.

Identification of Impurities

Impurities namely, 1-diaminomethylidene-3-methylguanidine hydrochloride (D1) and 1,3,5-triazine-2,4,6-triamine (D3), 2,4-thiazolidinedione (D11), 4-[2-(ethyl-pyridin-2-yl)-ethoxy]-benzaldehyde (D12) and 5-[4-[2-(5-ethyl-pyridin-2-yl)]-benzylidene] (D14) were suitably prepared in mobile phase at concentration of 100 µg/mL and spiked with injection of standards under optimised chromatographic conditions. The retention time of the

impurities was compared with the stress degradation products. It was identified that D1 and D3 belong to MET, whilst D11, D12 and D14 belong to PIO.

Table 1: Assay data for MET and PIO.

Drugs	Labelled claim (in mg)	Found (mean±SD)	Assay (%)
MET	330	331.36±1.75	100.4
PIO	10	9.89±0.97	98.90

RESULTS

Optimised Method

Chromatographic separation was achieved and optimised on C₈ column (Qualisil BDS 250 mm × 4.6 mm, 5 µm) with a mixture of methanol and water at 45:55 (v/v) containing 0.2% (w/v) HAS and 0.2% (v/v) TEA as mobile phase. The flow rate was 1 mL/min and eluents were detected at 265 nm. The optimised chromatogram was shown in Figure 3.

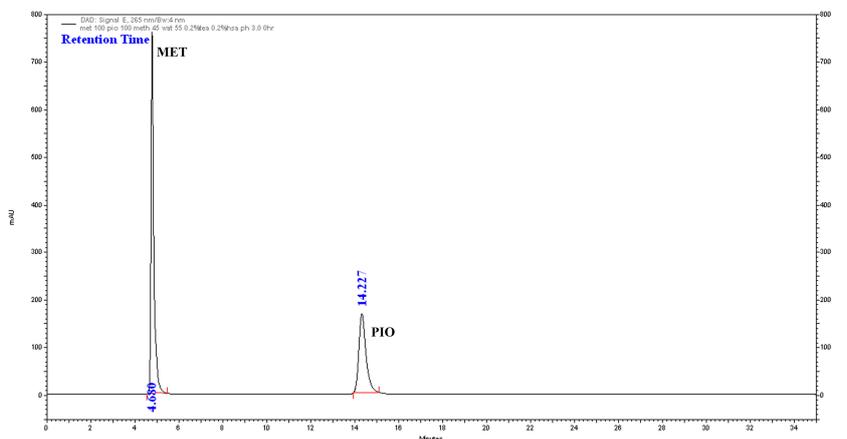


Fig. 3: Optimised RP-HPLC chromatogram of MET (retention time: 4.68) and PIO (retention time: 14.22) on C₈ column.

Validation of Results

The method was validated as outlined in ICH (Q2) guidelines with respect to specificity, linearity and range, accuracy, precision, robustness, limit of detection (LOD) and limit of quantification (LOQ).

Specificity

Forced degradation studies were performed on MET and PIO to support the specificity of the stability indicating method. The study was employed on degradation of MET and PIO by exposing to sunlight (for 70 h), heat (105°C for 6 h), acid hydrolysis [1N HCl, kept at room temperature (RT) for 48 h], base hydrolysis (0.1N NaOH, kept at RT for 48 h),

neutral (kept at RT for 10 days) and oxidation (3% H₂O₂, kept at RT for 10 days). All degradation products were adequately separated from MET and PIO, thus the specificity of the method was proven.

Linearity and Range

The linearity of detector response to different concentrations of MET and PIO was studied in the range from 100–750 µg/mL and 5–30 µg/mL, respectively for assay of formulations. Samples were analysed in triplicate at six different concentrations. The correlation coefficient (r^2 value) obtained was 0.9996 for MET and 0.9997 for PIO. However the linearity for stress studies was established between 50–200 µg/mL for both MET and PIO. The r^2 value obtained was 0.9995 for MET and 0.9997 for PIO.

Accuracy

Accuracy was performed by recovery studies using standard addition method. Standard drugs in the range of 80%, 100% and 120% of the sample concentrations were added into the sample solution as given in the Table 2. Each concentration was analysed in triplicate. Results of recovery studies were found to be in between 99.97% to 100.07% for MET and 98.18% to 100.08% for PIO.

Table 2: Accuracy data for MET and PIO.

Drug	Amount (µg/mL)	Recovery level (%)	Amount added (µg/mL)	Amount recovered (mean±SD, µg/mL)	% recovery (n=3)
MET	330	80	264	593.87±0.98	99.97
	330	100	330	660.47±1.72	100.07
	330	120	396	726.12±2.96	100.01
PIO	10	80	8	18.34±0.22	98.14
	10	100	10	20.16±0.18	100.8
	10	120	12	21.82±0.16	99.18

Precision

Data for intraday and interday precision studies were obtained from three different concentrations (100, 450, 750 µg/mL for MET and 5, 20, 30 µg/mL for PIO) in the linearity range. The % RSD values for intraday and interday precision were below 1.5%, indicating that the method was sufficiently precise and the result are shown in Table 3.

Limit of Detection and Limit of Quantification

LOD and LOQ were determined based on signal to noise ratio (S/N). The S/N ratio of 3:1 was taken as LOD and S/N of 10:1 was taken as LOQ. The LOD was found to be 0.464 µg/mL and 0.317 µg/mL while LOQ was 1.407 µg/mL and 0.962 µg/mL for MET and PIO, respectively.

Robustness

The robustness of the developed method was determined by analysing the samples under a variety of conditions of the method parameters, such as change in flow rate

(± 0.1 mL/min), pH (± 0.2) of the buffer and organic phase ($\pm 2\%$). The method was robust for all the parameters tested.

Table 3: Precision data for MET and PIO.

Drug	Amount ($\mu\text{g/mL}$)	Intraday (n=3)		Interday (n=3)	
		Found (mean \pm SD)	% RSD	Found (mean \pm SD)	% RSD
MET	100	87.22 \pm 0.588	1.22	89.85 \pm 0.71	1.61
	450	466.13 \pm 0.733	0.64	469.53 \pm 0.98	0.70
	750	748.82 \pm 2.512	0.28	749.45 \pm 3.83	0.33
PIO	5	5.16 \pm 0.25	1.31	5.30 \pm 0.05	1.88
	20	19.77 \pm 0.23	0.79	20.31 \pm 0.04	0.92
	30	29.65 \pm 1.50	0.52	29.77 \pm 1.72	0.76

Forced Degradation Studies

Degradation by Acid Hydrolysis

Initial degradation study was performed in 0.1N HCl, observed that the drug was stable. After that, degradation study was carried out in 1N HCl at room temperature for a period of 48 h. The drug degradation was 42.46% for MET and 28.91% for PIO in 48 h with 5 impurities i.e. D1 at 2.42, D4 at 3.92, D8 at 7.36 and D13 at 33.44 min formed as shown in Figure 4.

Base Induced Degradation

When the drug was exposed to 0.1N NaOH, the degradation was observed within 2 h and hence a milder condition of 0.1N NaOH was chosen as stress condition. The drug degradation was 30.43% for MET and 23.36% for PIO in 48 h with 4 impurities i.e. D2 at 2.80 min, D5 at 4.40 min, D7 at 6.48 min and D12 at 23.27 min as shown in Figure 5.

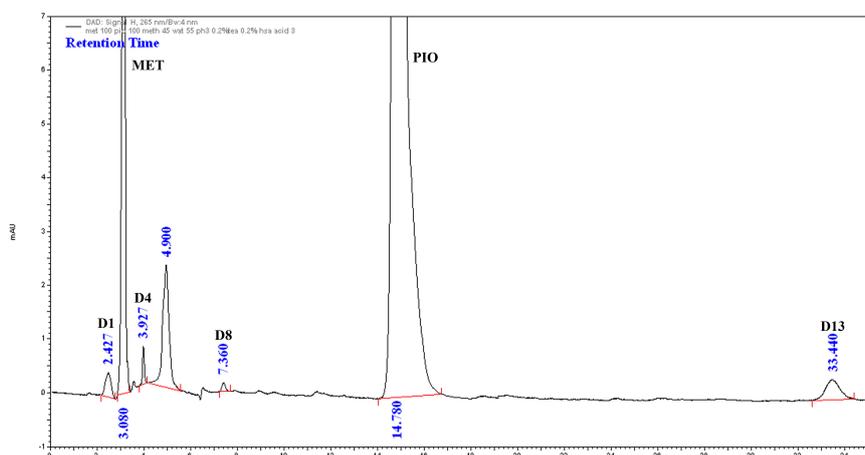


Fig. 4: Acid degradation (1N HCl for 48 h) chromatogram of MET and PIO.

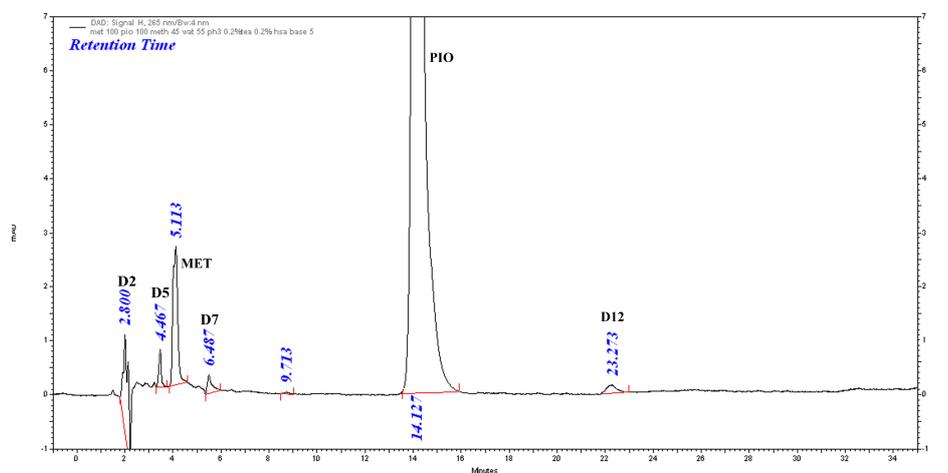


Fig. 5: Base degradation (0.1N NaOH for 48 h) chromatogram of MET and PIO.

Oxidative Degradation

Both MET and PIO showed negligible/no degradation in 0.3% H₂O₂ for 5 days and hence severe stress condition of 3% H₂O₂ was used. No degradation was observed for PIO, but 29.84% degradation was observed for MET and was optimised for specificity. There was one degradation product peak i.e. D1 at 2.42 min, as shown in Figure 6.

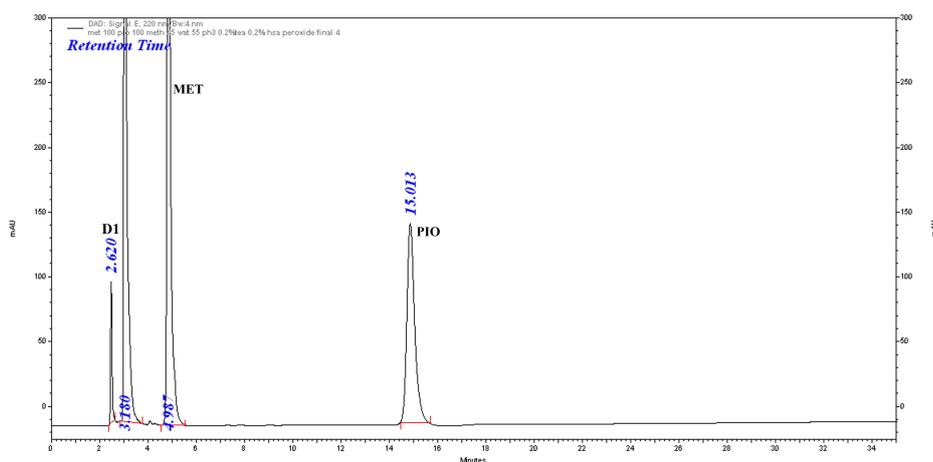


Fig. 6: Oxidative degradation (3% H₂O₂ for 10 days) chromatogram of MET and PIO.

Thermal Degradation

When drugs were exposed to dry heat in oven at 105°C for 6 h, 7 degradation products were formed at 3.20 min (D3), 3.78 min (D4), 4.40 min (D5), 5.64 min (D6), 8.13 min (D9), 11.66 min (D11) and 34.19 min (D14) with significant changes in the peak area of the

parent drug. Drugs MET showed 69.75% and PIO 67.80%. These data are shown in Figure 7.

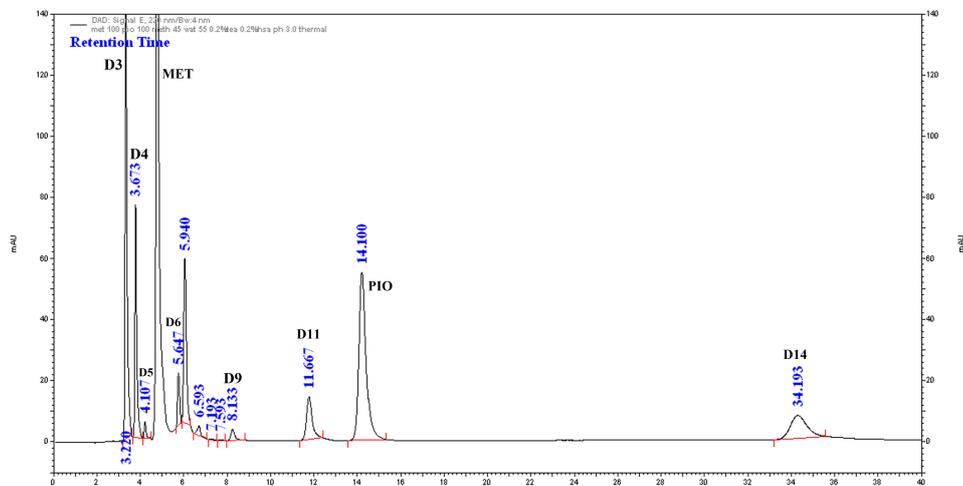


Fig. 7: Thermal degradation (solid – 105°C for 6 h) chromatogram of MET and PIO.

Photolytic Degradation

When drugs were exposed to photolytic degradation in sunlight for 70 h, 16.95% and 24.34% degradation were observed for MET and PIO, respectively. Four degradation products were formed at 3.20 min (D3), 3.78 min (D4), 4.40 min (D5) and at 11.66 min (D11) as shown in Figure 8.

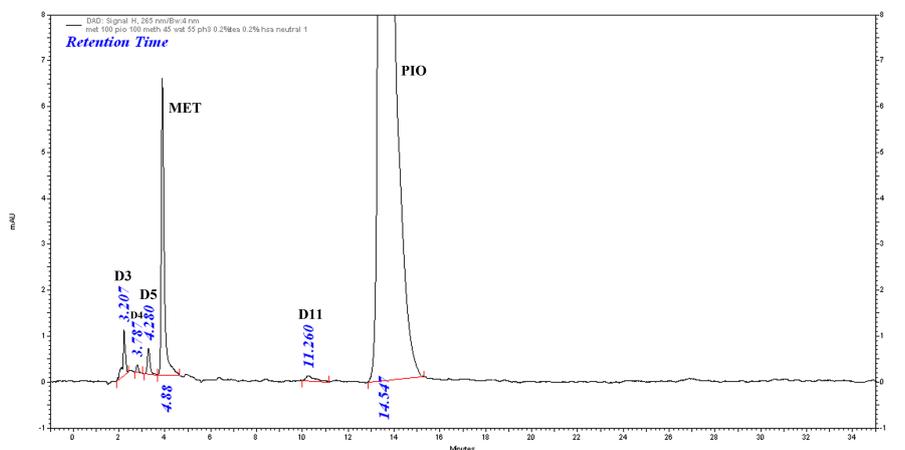


Fig. 8: Photo degradation (solution – sunlight for 70 h) chromatogram of MET and PIO.

Neutral Degradation

The drug degradation of 20.69% for MET and 10.62% for PIO was observed after 10 days at room temperature (22°C) with a total of 4 impurities i.e. D5 at 4.40, D8 at 7.36, D10 at 9.46 and D11 at 11.66 min, as shown in Figure 9.

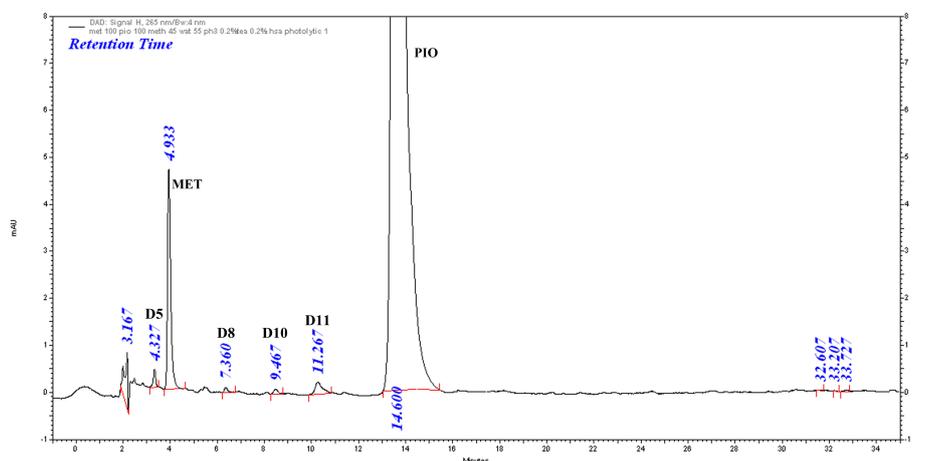


Fig. 9: Neutral degradation (in methanol for 10 days) chromatogram of MET and PIO.

DISCUSSION

The objectives of the present chromatographic method were to separate both MET and PIO from all their possible degradation products and to elute them as symmetrical peaks. Agilent Qualisil BDS column C₈ was used as stationary phase with a flow rate of 1 mL/min and PDA detection wavelength was fixed at 265 nm. Various trials with methanol and water as mobile phase (80:20, v/v to 50:50, v/v) were performed. It was found out that MET was not retained, whereas PIO was observed in between 5–20 min with a tailing factor of more than 2.5. The use of HSA at 0.2% in aqueous part of mobile phase retained MET but tailing was observed. To reduce the tailing effects, several trials were carried out by varying pH from 3.0 to 6.0, but it was found ineffective. The use of TEA between 0.10%–0.25% in aqueous phase with pH to 3.0 with OPA was found to reduce the tailing and enabled separation of MET and PIO with adequate resolution, theoretical plate and retention for SIAM. Therefore, 0.2% of TEA and 0.2% HSA was used in optimised condition. The retention time of MET and PIO was 4.68±0.05 and 14.22±0.4 min, respectively. The method has proven specificity by separating the degradation products in various stress conditions. It was observed that 14 major degradation products were formed with retention times of 2.42±0.2 (D1), 2.80±0.22 (D2), 3.78±0.11 (D3), 3.92±0.14 (D4), 4.40±0.23 (D5), 5.64±0.31 (D6), 6.48 (D7), 7.36 (D8), 8.13 (D9), 9.46 (D10), 11.66±0.41 (D11), 23.27 (D12), 33.44 (D13) and 34.19 min (D14). The % degradation and number of degradation products formed were detailed in Table 4.

Table 4: Comparative study of stability data of MET and PIO (control and combined stress).

Conditions (duration)	% degradation				Impurities (Dx) (x = 1–15)			
	Control		Combined		Control		Combined	
	MET	PIO	MET	PIO	MET	PIO	MET	PIO
Methanol at RT (10 days)	3.14	5.54	20.69	10.62	2	8, 11	2, 5	8,10, 11
1N HCl (48 h)	25.16	18.14	42.46	28.91	1, 2 ,4	8, 13	1, 2, 4	8, 13
0.1N NaOH (48 h)	29.24	16.65	30.43	23.36	2, 5	7, 9	2,5	7, 12
3% H ₂ O ₂ (10 days)	19.58	0.00	29.84	0.00	1	–	1	–
Thermal 105°C (6 h)	47.34	42.16	69.75	67.80	2,3, 5	3, 6, 9, 11	2, 3, 5	6, 9, 11, 14
Photolytic (70 h)	9.95	8.34	16.95	24.34	2,3,5	11	2,3,5	11

Notes: Degradation product retention time (min): 2.42±0.2 (D1), 2.80±0.22 (D2), 3.78±0.11 (D3), 3.92±0.14 (D4), 4.40±0.30 (D5), 5.64±0.31 (D6), 6.48 (D7), 7.36 (D8), 8.13 (D9), 9.46 (D10), 11.66±0.4 (D11), 23.27 (D12), 33.34 (D13), 34.19 (D14)

The study revealed that MET was more sensitive for all stress conditions whereas PIO remained affected under all conditions that is acidic, basic, photolytic, neutral and thermal degradations except, oxidative stress. The peak purity of both MET and PIO was more than 0.999 in all stress conditions investigated, no degradation product was observed after 40 min. A total of 14 degradation products (D1–D14) were detected in the present study, among them are structures of D1, D3, D11, D12 and D14 as identified by Spike analysis (ICH 2007) (Fig. 10). 1-(diaminomethylidene)-3-methylguanidine hydrochloride (D1), 1,3,5-triazine-2,4,6-triamine (D3), 2,4-thiazolidinedione (D11), 4-[2-(ethyl-pyridin-2-yl)-ethoxy]-benzaldehyde (D12) and 5-[4-[2-(5-ethyl-pyridin-2-yl)]-benzylidene] (D14) were suitably prepared in mobile phase at concentration of 100 µg/mL and spiked with injection of standards under optimised chromatographic conditions. Retention time of the impurities was compared with the stress degradation products. It was identified that D1 (2.42 min) and D3 (3.78 min) belong to MET, whilst D11 (11.66 min), D12 (23.27 min) and D14 (34.19 min) belongs to PIO. Thermal degradation yielded 69.75% product for MET while PIO produced 67.80%. PIO was found to be highly stable in methanol. Degradation products details were shown in Table 4. Although conditions were same for MET and PIO stress, individually and combined, impurities D6, D9 and D14, were detected in combination and 20% excess in total degradation of PIO was observed under thermal conditions of 105°C for 6 h.

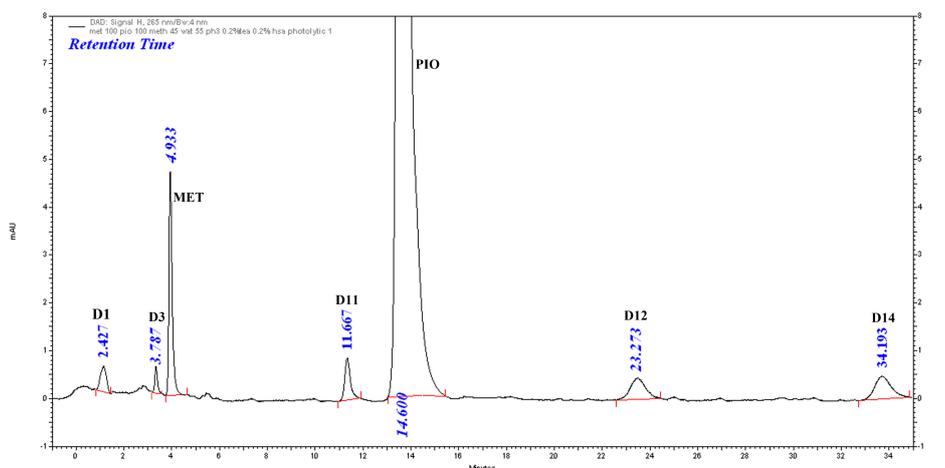


Fig. 10: Typical chromatogram of MET, PIO and spiked impurities.

The percentage degradation for both drugs was significantly enhanced in combined stress, compared to their control. PIO remained unaffected by the peroxides in both control and combined stress. It may be due to the interaction or secondary degradation products formed due to enhanced degradations. To conclude, the results of stress testing studies indicate a high degree of specificity of this method for both MET and PIO. These results also suggest the need for simultaneous stability for dosage forms, to reveal the new degradation products and rate of degradation.

CONCLUSION

The method was validated for all validation parameters as per ICH guidelines. The linearity range for MET and PIO was 100–750 µg/mL and 5–30 µg/mL with r^2 value of 0.9996 and 0.9997 respectively. The % RSD for intra-day precision was <2%. The developed and validated stability indication HPLC method is found to be linear, accurate, precise, specific and robust, confirming the stability indicating method for the simultaneous estimation of MET and PIO in presence of identified impurities D1, D3, D11, D12 and D14 or all possible degradation products.

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