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 method was developed for simultaneous analysis of Metformin hydrochloride (MET) and Pioglitazone hydrochloride (PIO) in dosage forms. Chromatographic separation was performed on C_8 column (Qualisil BDS 250 mm x 4.6 mm, 5 µm) with a mixture of methanol and water at 45 : 55 (v/v) containing 0.2 % (w/v) n -heptanesulfonicacid , 0.2 % (v/v) Triethyl amine 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 r^2 value of 0.9996 and 0.9997. Drugs were subjected to acidic and basic hydrolysis, oxidative, photolytic, neutral and thermal degradation. This method revealed 14 degradants and among these products D1, D3, D11, D12 and D14 were identified using impurity standards.

***Keywords**:* RP-HPLC, Stability indicating, Metformin HCL, Pioglitazone HCL

INTRODUCTION

Metformin hydrochloride (MET) chemically, 3-(diaminomethylidene)-1, 1-dimethylguanidine hydrochloride is used 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. Sun Pharmaceutical Industries Ltd. markets PIO in combination forms as Pioglit-MF. In combination these are available in 15 mg : 500 mg of PIO and MET, respectively.

Pioglitazone hydrochloride (PIO) is chemically [(±)-5-[[4-[2-(5-ethyl-2- pyridinyl) ethoxy] phenyl] methyl] -2, 4-] thiazolidinedione monohydrochloride Fig. 2. It is used in the management of type 2 diabetes mellitus (also known as non-insulin-dependent diabetes mellitus [NIDDM] or adult-onset diabetes). Pharmacological studies indicate that pioglitazone hydrochloride improves sensitivity to insulin in muscle and adipose tissue and inhibits hepatic gluconeogenesis.

Literature reveals that various methods like UV (Sujana, Swathi and Bhanu Prasad et al., 2010; Ajithdas and nancy 2000), HPLC (Srinivas, Venkataramana and Srinivasa Rao et al., 2012; Jeyabalan and Nyola 2012; Lad, Bhoir and Bhoir et al., 2003) and ion-pair HPLC (Vasudevan, Ravi and Ravisankar et al., 2001) method have been reported for the estimation of MET and PIO alone, along with other drugs in various dosage forms. PIO is not yet official in any of the pharmacopoeia but MET is official in IP (Indian Pharmacopoeia 1996; British Pharmacopoeia 2002 and United State Pharmacopoeia – NF 2005). Literature survey reveals that few bio-analytical LC-MS (Xue, Turner and Meeker et al., 2003; Yamashita, Murakami and Okuda et al., 1996; Lin, Ji and Desai-Kriger et al., 2003) 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 prompted to pursue the present work. The main objective of this present work is to develop and validate a stability indicating reverse phase high performance liquid chromatographic method for the simultaneous estimation of MET and PIO that could demonstrate all possible impurities in combined dosage forms. Here in we report a stability-indicating RP-HPLC method for simultaneous determination of MET and PIO in the presence of their degradation products as per ICH guidelines.

EXPERIMENTAL

Chemicals and reagents

Samples of MET and PIO were obtained as gift sample from HeteroLabs, Hyderabad Pvt. Ltd (India). Tablet formulation of Metformin hydrochloride and pioglitazone hydrochloride (Pioglit-MF tablet) were procured from commercial market (Sun Pharmaceutical Industries Ltd.). All the solutions were protected from light and were analyzed on the day of preparations. Glasswares used in each procedure were soaked overnight in a mixture of

chromic acid and sulphuric acid rinsed thoroughly with double distilled water and dried in hot air oven. Water and Methanol (HPLC grade) were purchased from Merck, India.

HPLC instrumentation and conditions

HPLC analysis for method development, forced degradation studies, and method validation was performed with an Agilent-1200 binary pump plus manual sampler and Agilent photo diode-array detector (PDA). The output signal was monitored and processed using Ezchrome elite software resident in a Pentium computer (Digital Equipment). Compounds were separated on a 250 mm x 4.6 mm, 5 µm particle, Agilent C_8 column with methanol and water (45 : 55, *v/v*) with 0.2 % (*w/v*) n-heptanesulfonicacid (HSA), 0.2 % (*v/v*) Triethyl amine (TEA) and pH was adjusted to 3.0 with ortho phosphoric 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 substance 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*. pH of stress sample was adjusted to 3-4 and injected in the optimized condition with appropriate blank. The samples from acid hydrolysis were neutralized with 0.1N NaOH and the samples from base hydrolysis were neutralized 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 peak area.

Preparation of Stock Solution for stress studies

An accurately weighed quantity of 10 mg of each drug substance was carefully transferred in to 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 % H_2O_2. Thermal degradation was carried out for solid state by means of heating the samples over a period in hot air oven, at 105 ^oC. Photo degradation was carried on solution

sample as per outlined procedure in the following section. In all stress studies, stress was carried out for both MET and PIO alone (control) as well as in combined form. Result of degradation studies for MET and PIO alone (control) was 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. 1mL volume of sample was 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 neutralized with 0.1N NaOH and the samples from base hydrolysis were neutralized 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 in to 10 mL volumetric flask and made to 10 mL with mobile phase and injected into the optimized conditions at various time intervals against blank.

Thermal degradation

For, Solid state stability, it was performed on preheated sample as thin layer in the petridish at 105 ºC. At various time intervals (0, 3, 6 h), 10 mg of the heated samples were weighed, suitably dissolved and diluted with mobile phase to get a concentration of 100 *µg/mL* and injected into the system.

Photo degradation

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

Preparation of sample solution for assay

Twenty tablets of marketed formulation Pioglit-MF (Sun Pharmaceutical Industries Ltd.) containing PIO 15 mg and MET 500 mg formulation 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 mins with methanol and volume was made up to 100 mL with diluent. 0.1 mL of above solution was taken in 10 mL volumetric flask and volume was made up to 10 mL with mobile phase, and final solution was filtered through 0.45 µ syringe filter and it was analysed. The results of the assay were shown in Table 3.

Identification of Impurities

Impurity 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-[-(-ethyl-pyridin-2-yl]-benzyldiene(D14) were suitably prepared in mobile phase at concentration of 100 *µg/mL* and spiked with injection of standards under optimized chromatographic conditions. Retention time of the impurities was compared with the Stress degradation products. It was identified that D1, D3 belongs to Metformin, whilst D11, D12, D14 belongs to Pioglitazone.

RESULTS

Optimized method

Chromatographic separation was achieved and optimized on C_8 column (Qualisil BDS 250 mm x 4.6 mm, 5 µm) with a mixture of methanol and water at 45 : 55 *(v/v)* containing 0.2 % *(w/v)* n -heptanesulfonicacid , 0.2 % *(v/v)* Triethyl amine as mobile phase. The flow rate was 1 mL/min and eluents were detected at 265 nm. The optimized chromatogram was shown in Fig.3.

Validation Results

Method was validated as per ICH (Q2) guidelines with respect to specificity, linearity and range, accuracy, precision, robustness, limit of detection and limit of quantification [14].

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 sun light (for 70 h), heat (105 ^{0}C for 6 h), acid hydrolysis (1 N HCl, kept at RT for 48 h), base hydrolysis (0.1 N NaOH, kept at RT for 48 h), neutral (kept at RT for 10days) and oxidation (3% H_2O_2, kept at RT for 10 days). All degradants 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 linearity for stress studies was established between 50-200 *μg/mL* for both MET and PIO. The correlation coefficient (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 concentration were added into the sample solution as given in the Table 1. 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.

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 is shown in Table 2.

Limit of detection and limit of quantification

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

Robustness

The robustness of the developed method was determined by analyzing 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 (±2 %). The method was robust for all the parameters tested.

Forced Degradation Studies

Degradation by acid hydrolysis

Initial degradation study was performed in 0.1N HCl, observed that the drug was stable. Upon treatment with 1N HCl. The drug degradation was 42.46 % for MET and 28.91 % for PIO in 48 h with five impurities i.e. D1 at 2.42, D4 at 3.92, D8 at 7.36 and D13 at 33.44 min were formed as shown in Fig. 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 four 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 Fig. 5.

Oxidative degradation

Both MET and PIO showed negligible / no degradation in 0.3 % H_2O_2 for 5 days and hence severe stress condition of 3 % H_2O_2 was used, no degradation was observed for PIO, but 29.84 % degradation was observed for MET and was optimized for specificity. There were one degradant peak i.e. D1 at 2.42 min, as shown in Fig. 6.

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 at 34.19 min (D14) with significant change in peak area of the parent drug. The MET showed 69.75 % and PIO showed 67.80 % of degradation, as shown in Fig.7.

Photolytic degradation

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

Neutral Degradation

The drug degradation was 20.69 % for MET and 10.62 % for PIO was observed after 10 days at room temperature. A total five impurities i.e. D5 at 4.40 min and D8 at 7.36, D10 at 9.46 and D11 at 11.66 min as shown in Fig. 9.

DISCUSSION

The objectives of the present chromatographic method were to separate both MET and PIO from their all possible degradants and to elute them as symmetrical peak. Agilent Qualisil BDS column C_8 (250 mm × 4.6 mm, 5 µm) was used as stationery phase. The flow rate was 1

mL/min and the photo diode array detection wavelength was 265 nm. Various trials with methanol and water as mobile phase (80 : 20 *v/v* to 50 : 50 *v/v*) were performed, and MET was not retained and PIO was observed in between 5 - 20 min with tailing factor more than 2.5. Use of heptane sulfonic acid (HSA) at 0.2 % in aqueous part of mobile phase retained MET but tailing was observed. For reducing tailing effect several trials were carried out by varying pH from 3.0 to 6.0, result was ineffective. It was found that us of Triethyl amine (TEA) between 0.25 % in aqueous phase with pH to 3.0 with ortho phosphoric acid (OPA) reduced the tailing and enabled separation of MET and PIO with adequate resolution, theoretical plate and retention for stability indicating assay method. 0.2% of TEA and 0.2 % HSA was used in optimized condition. The retention time of MET and PIO was 4.68 ±0.05 min and 14.22 ± 0.4 min, respectively. The method has proven specificity by separating the degradants in various stress conditions. It was observed that fourteen major degradants were formed with retention times of 2.42±0.2 min (D1), 2.80±0.22 min (D2), 3.78±0.11 min (D3), 3.92±0.14 min (D4), 4.40±0.23 min (D5), 5.64±0.31 min (D6), 6.48 min (D7), 7.36 min (D8), 8.13 min (D9), 9.46 min (D10), 11.66±0.41 min (D11), 23.27 min (D12), 33.44 min (D13), 34.19 min (D14). The %degradation and number of degradants formed were detailed in table 4.

The study revealed that MET was more sensitive for all stress conditions whereas PIO was remained affected all conditions acidic, basic, photolytic, neutral and thermal except, oxidative stress. The peak purity of both MET and PIO was more than 0.999 in all stress conditions investigated, no degradant was observed after 40 min. A total of 14 degradants (D1 – D14) were detected in the present study, among them, structures of D1, D3, D11, D12, D14 were identified by Spike analysis 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-[-(-ethyl-pyridin-2-yl]-benzyldiene(D14) were suitably prepared in mobile phase at ac concentration of 100 *µg/mL* and spiked with injection of standards under optimized chromatographic conditions. Retention time of the impurities was compared with the Stress degradation products. It was identified that D1 (2.42 min), D3 (3.78 min) belongs to Metformin, whilst D11 (11.66 min), D12 (23.27 min), D14 (34.19 min) belongs to Pioglitazone. Thermal degradation yielded 69.75 % product for MET while PIO produced 67.80 %. PIO was found to be highly stable in methanol. Degradants details were shown in Table 4. Although conditions were same for MET and PIO stress, alone and combined, impurities

D6, D9, D14, were detected in combination and 20% excess in total degradation of PIO was observed under thermal conditions of 105 ° C for 6 h.

The percentage degradation for both drugs was significantly enhanced in combined stress, compared to their control. PIO was remained unaffected by the peroxides in both control and combined stress. It may be due to the interaction or secondary degradants 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

A simple, specific stability indicating RP-HPLC method was developed for the estimation of Metformin hydrochloride (MET) and Pioglitazone hydrochloride (PIO) in pharmaceutical dosage form and validated according to ICH Q2 (R2) guidelines. The method was found to be specific for the detection of all possible impurities in the dosage form under various conditions and accurate, precise and robust for the assay of MET and PIO in dosage forms. Confirming the stability indicating method for the simultaneous estimation of MET and PIO in presence of identified impurities D1, D3, D11, D12, D14, / all possible degradation products.

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Table 1: Accuracy.

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

Table 2: Precision.

Drug	Amount (*µg/mL*)	Intraday (n=3)		Interday(n=3)	
		Amount found Mean ± SD	% RSD	Amount found Mean ±SD	% RSD
	100	87.22 ± 0.588	1.22	89.85 ± 0.71	1.61
MET	450	466.13 ± 0.733	0.64	469.53 ± 0.98	0.70
	750	748.82 ± 2.512	0.28	749.45 ± 3.83	0.33
	5	5.16 ± 0.25	1.31	5.30 ± 0.05	1.88
PIO	20	19.77 ± 0.23	0.79	20.31 ± 0.04	0.92
	30	29.65 ± 1.50	0.52	29.77 ± 1.72	0.76

Table 3: Assay.

Formulation	Drugs	Labeled Claim (in mg)	Amount Found (Mean ± SD)	Assay (in %)
A	MET	330	331.36 ± 1.75	100.4
	PIO	10	9.89 ± 0.97	98.90

Table 4: Comparative study of stability data of MET and PIO: Control and Combined stress.

Conditions (Duration)	% degradation				No. of Impurities[a] (D x) (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_2O_2 (10 days)	19.58	0.00	29.84	0.00	1	--	1	--
Thermal 105ºC (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: Degradants Rt: 2.42±0.2 min (D1), 2.80±0.22 min (D2), 3.78±0.11 min (D3), 3.92±0.14 min (D4), 4.40±0.30 min (D5), 5.64±0.31 min (D6), 6.48 min (D7), 7.36 min (D8), 8.13 min (D9), 9.46 min (D10), 11.66±0.4 min (D11), 23.27 min (D12), 33.34 min (D13), 34.19 min (D14).

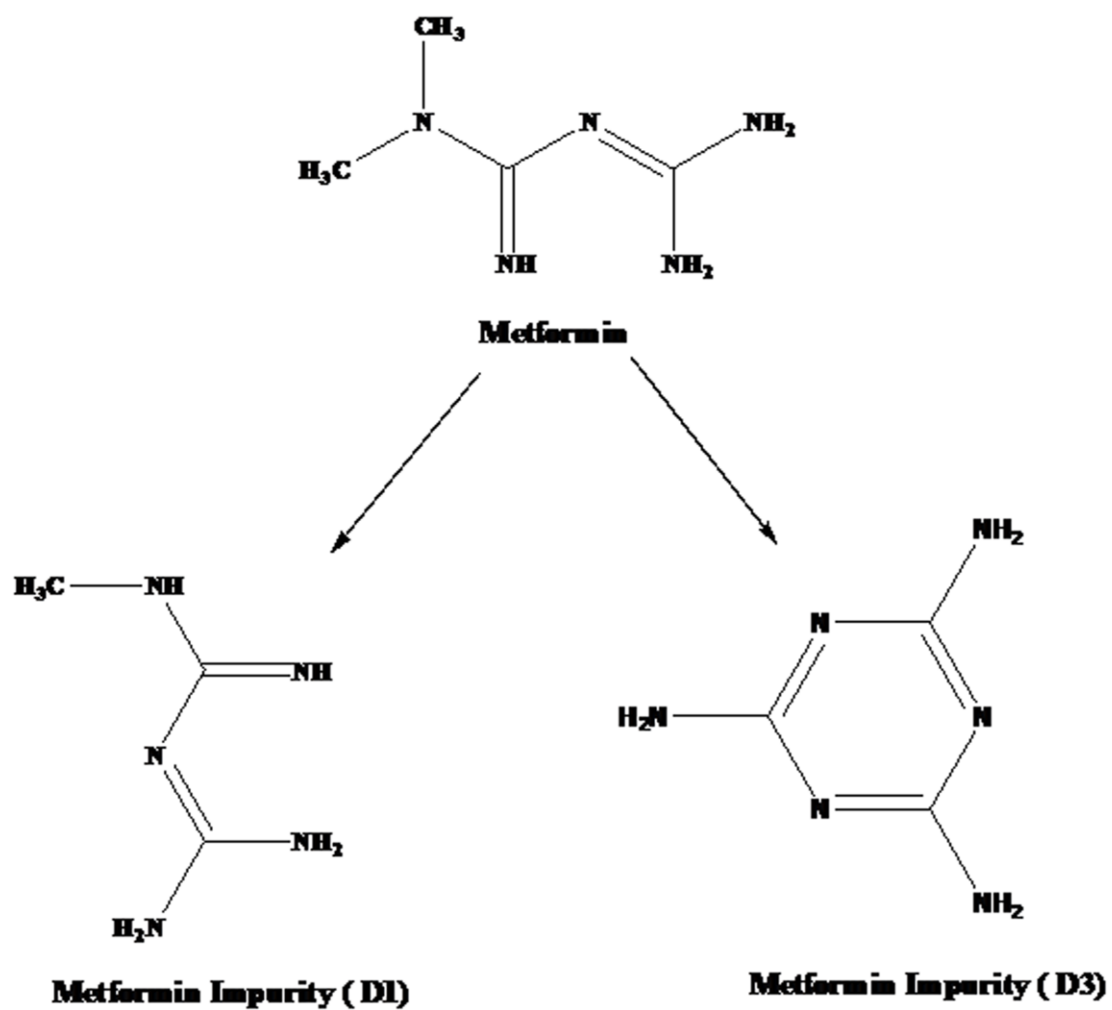

Fig. 1: Structures of Metformin Hydrochloride, Impurity-D1 and Impurity-D3.

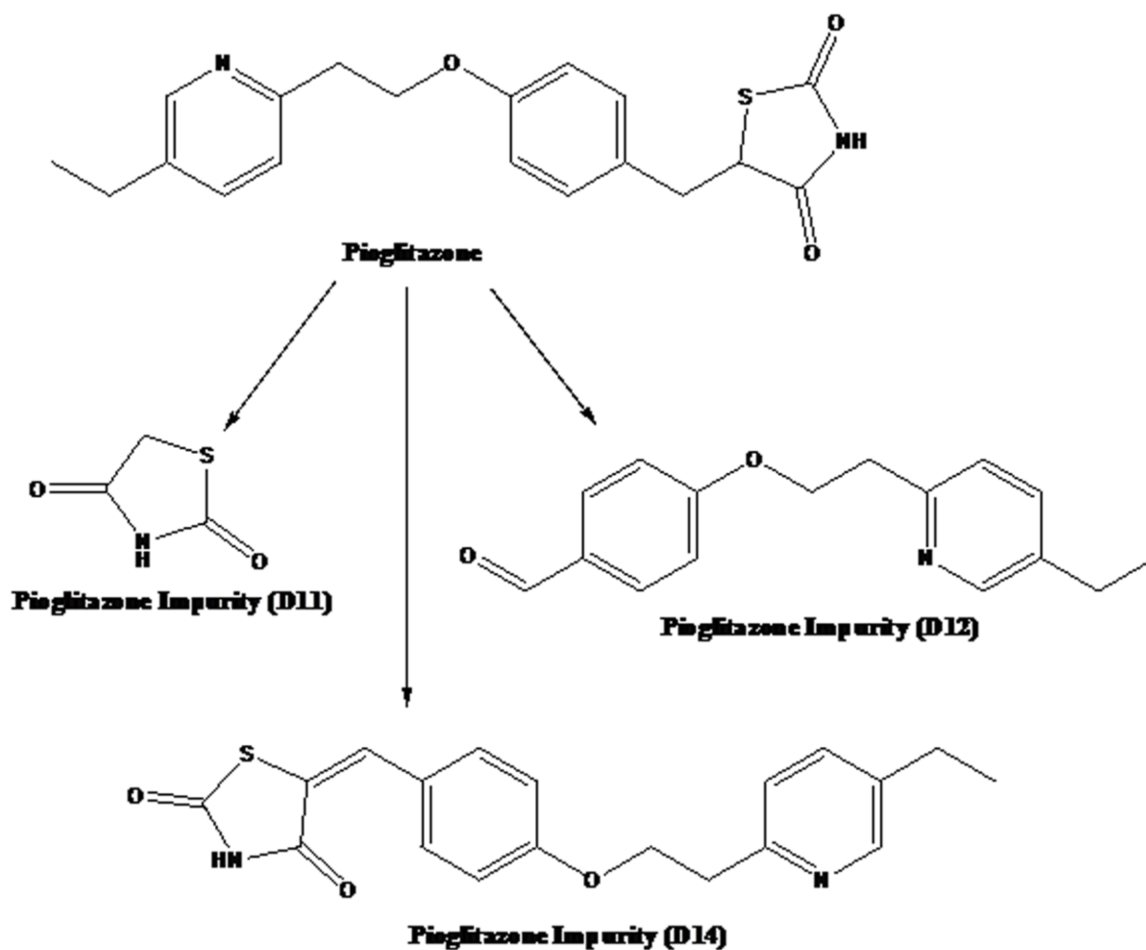

Fig. 2: Structures of Pioglitazone HCL, Impurity-D11, Impurity-D12 and Impurity-D14.

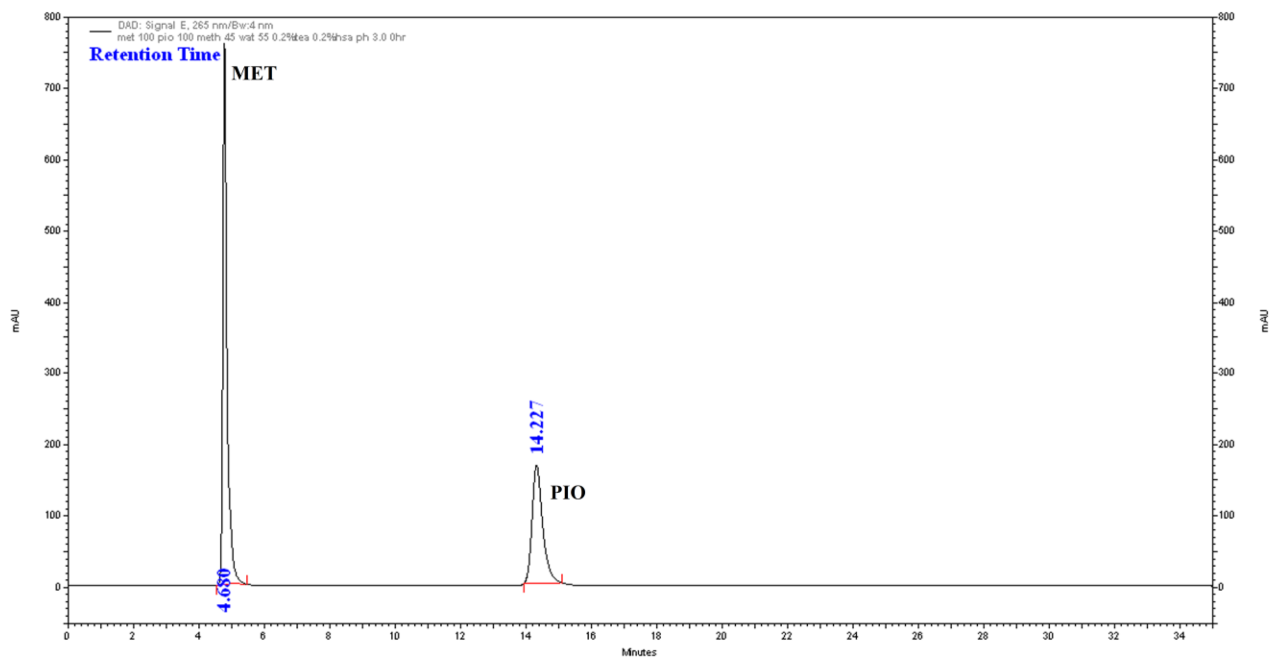

Fig. 3: Optimised RP-HPLC Chromatogram of MET (Rt: 4.68) and PIO (Rt: 14.22) on C_8 Column.

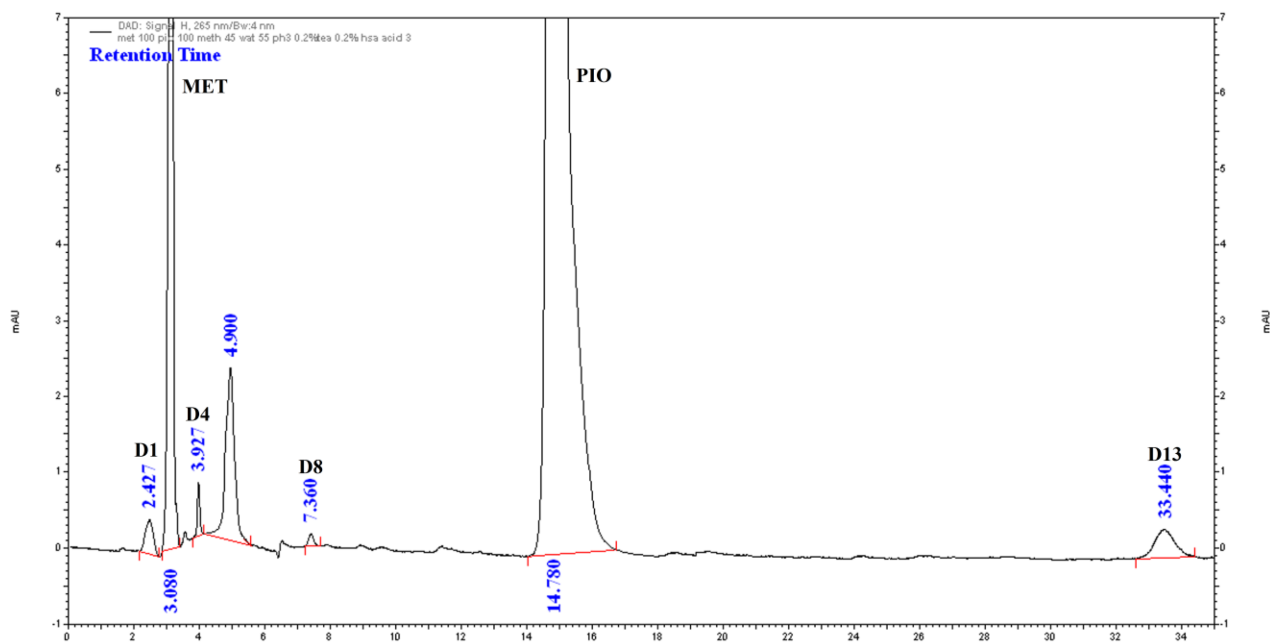

Fig. 4: Acid degradation (1N HCl for 48 h) chromatogram of MET and PIO.

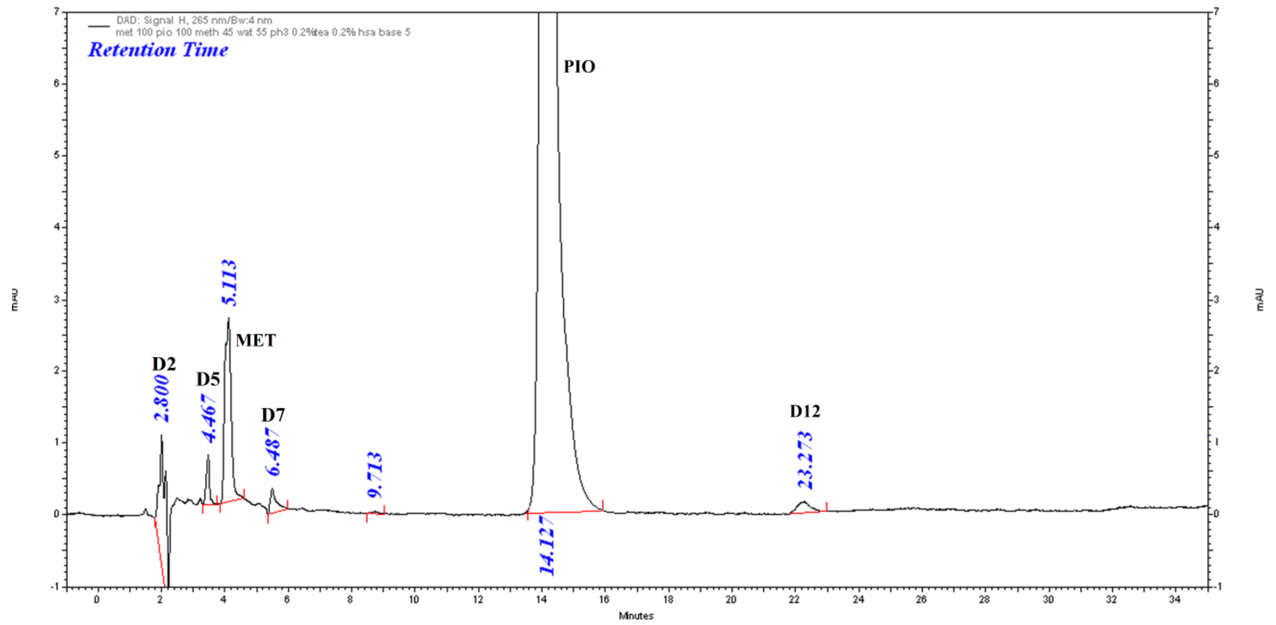

Fig. 5: Base degradation (0.1N NaOH for 48 h) chromatogram of MET and PIO.

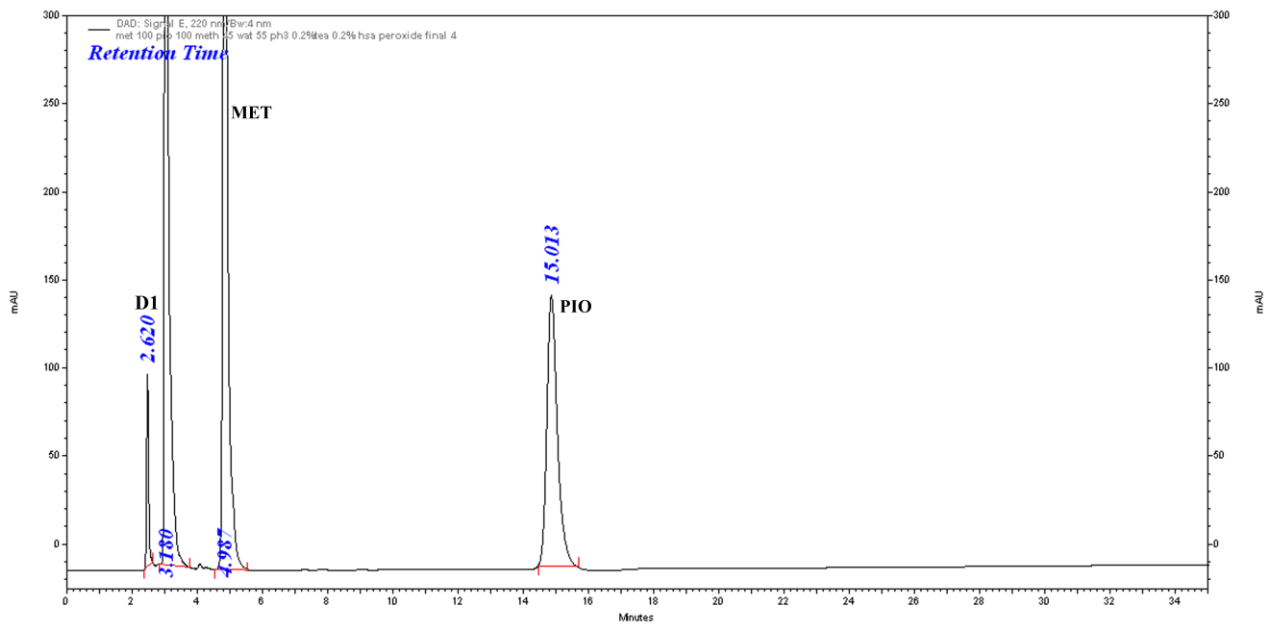

Fig. 6: Oxidative degradation (3 % H_2O_2 for 10 days) Chromatogram of MET and PIO.

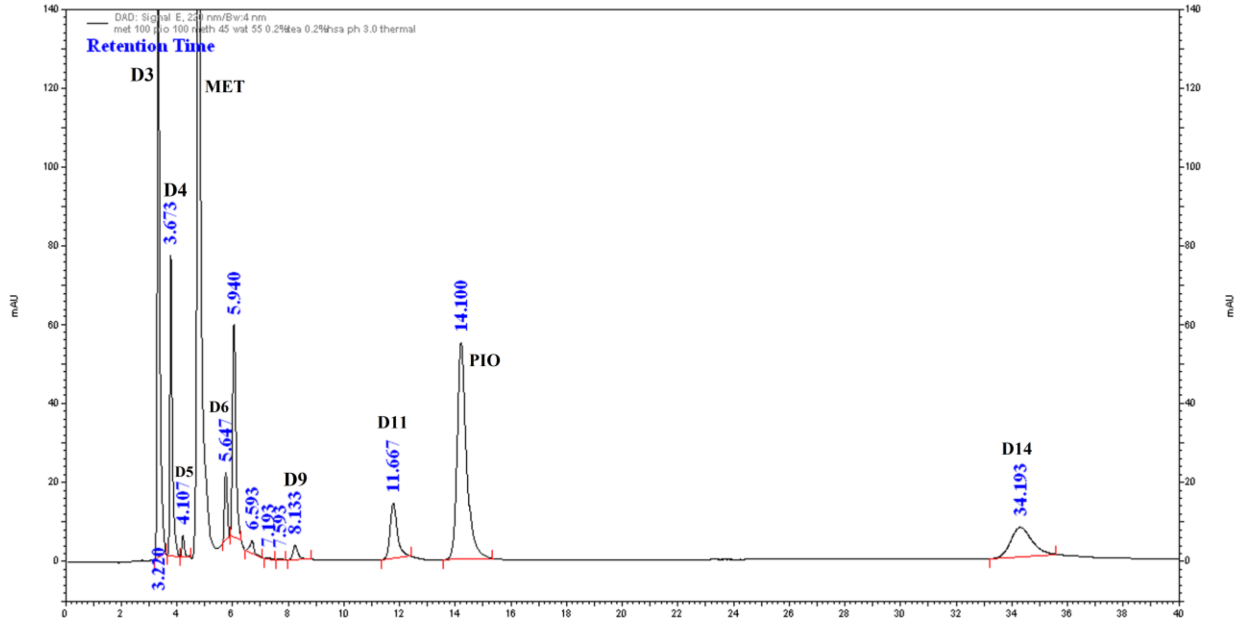

Fig. 7: Thermal degradation (Solid - 105º C for 6 h) chromatogram of MET and PIO.

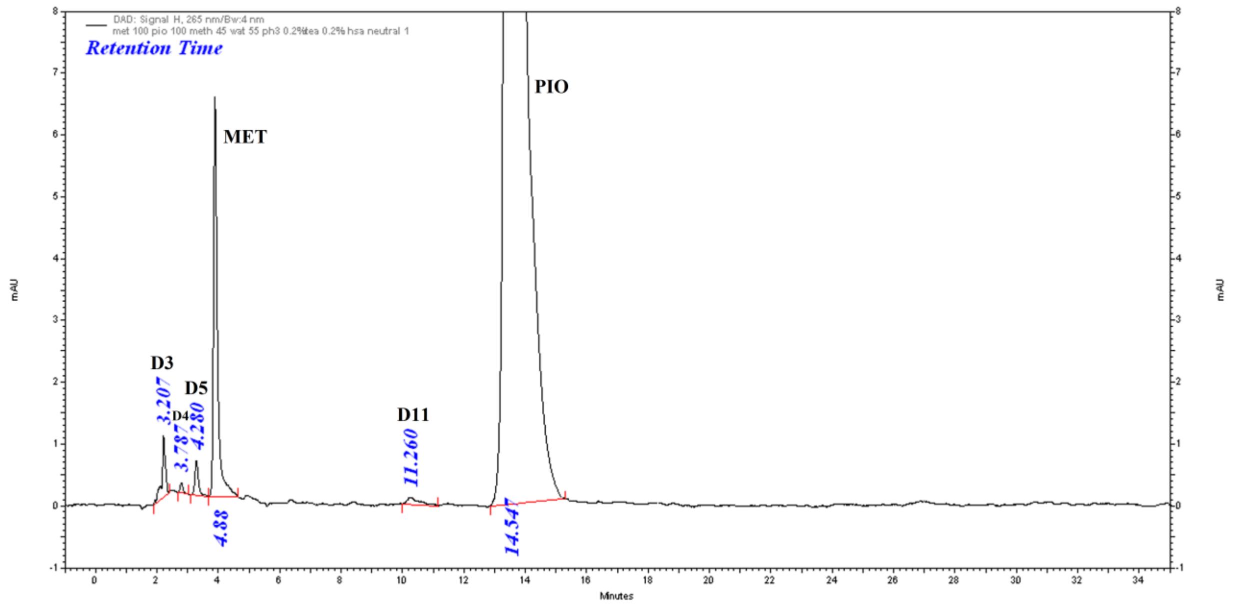

Fig. 8: Photo degradation (solution – sunlight for 70 h) chromatogram of MET & PIO.

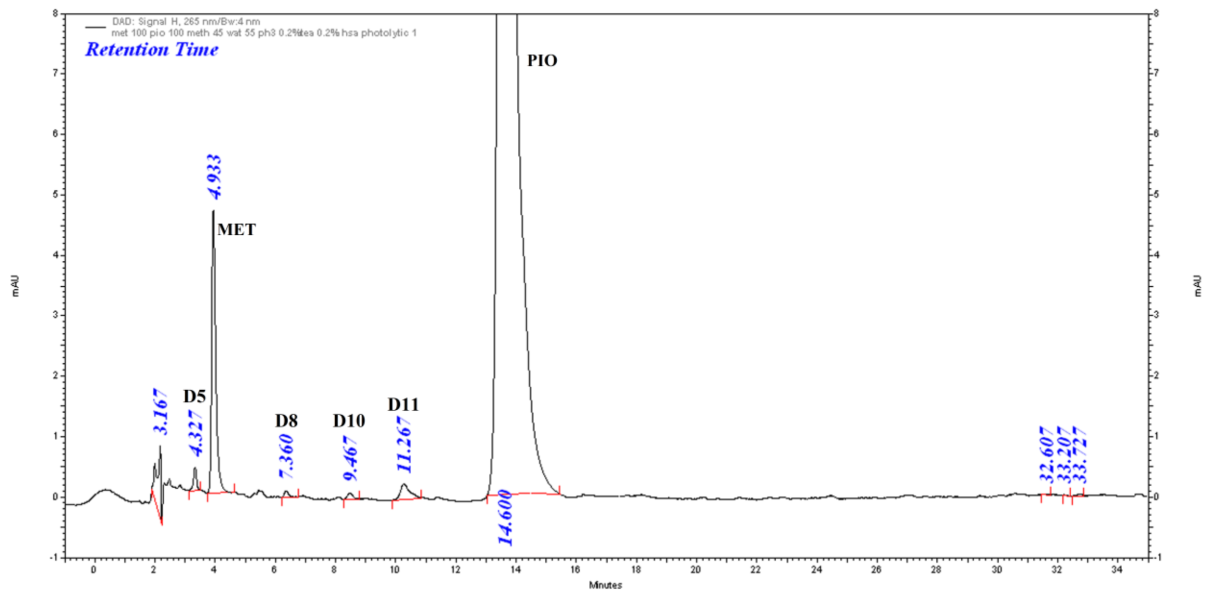

Fig. 9: Neutral degradation (in Methanol for 10 days) chromatogram of MET and PIO.

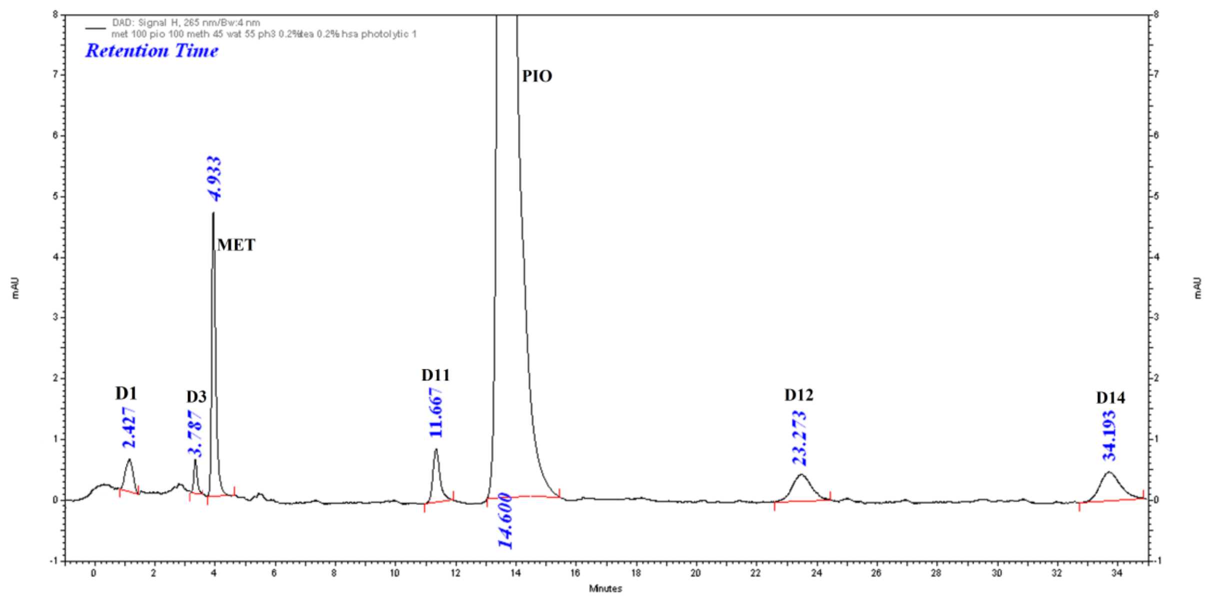

Fig. 10: Typical chromatogram of MET, PIO and spiked impurities.