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STRESS DEGRADATION BEHAVIOR OF PREGABALIN, IDENTIFICATION OF DEGRADATION IMPURITIES AND DEVELOPMENT OF STABILITY INDICATING UPLC METHOD

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Keywords:

Pregabalin, Ultra performance liquid chromatography (UPLC), Stability-indicating methods, Identification of forced degradation impurities by NMR & Mass Validation

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ABSTRACT: A novel, high-throughput, reverse phase-ultra performance liquid chromatographic (RP-UPLC) method has been developed for the quantification of Pregabalin and its related impurities in drug substance. The stability-indicating capability of the developed method is demonstrated using forced degradation samples from stress conditions such as hydrolysis, oxidation, thermal and photolytic degradation. The separation of known impurities and degradation impurities are accomplished using a phenyl-hexyl stationary phase with 100 mm length and 1.7 µm particle size in short run time (10 min). The developed method employs a linear gradient elution with phosphate buffer pH 6.2-acetonitrile as mobile phase, and is validated in accordance with International Conference on Harmonization requirements. During forced degradation it has been observed significant degradation of drug substance in base hydrolysis and oxidative stress conditions, and slight degradation of drug substance in acid hydrolysis and thermal stress conditions. The ten major oxidative degradation impurities formed are identified by LC-MS, HR-MS and NMR techniques and the proposed structure are reported.

INTRODUCTION: Pregabalin (S)-(+)-3-(aminomethyl)-5-methylhexanoic acid [1-3] is an anticonvulsant drug used for neuropathic pain and as an adjunct therapy for partial seizures with or without secondary generalization in adults. Pregabalin binds to the α2δ (alpha2delta) subunit of the voltage-dependent calcium channel in the central nervous system and decreases the release of neurotransmitters including glutamate, norepinephrine, substance P and calcitonin gene related peptide, reducing the communication between nerves can contribute to the effect on pain and seizures.

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Pregabalin, a gamma-aminobutyric acid analogue, is a prescription drug sold under the trade name Lyrica®. The maximum recommended dose is 100 mg three times a day (300 mg/day) in patients with creatinine clearance of at least 60 mL min^{-1}. Begin dosing at 50 mg three times a day (150 mg/day). The dose may be increased to 300 mg/day within 1 week based on efficacy and tolerability. Pregabalin is eliminated primarily by renal excretion; adjust the dose in patients with reduced renal function. In the last years, a misuse of Pregabalin has been reported in Germany [4-5] as well as in other countries like Sweden [6].

In the USA, Pregabalin is listed as a substance with potential abuse [7]. In order to monitor a potentially increasing abuse of Pregabalin, it was decided to analyze routinely for Pregabalin in postmortem toxicology [8] at the institute of forensic medicine in Munich.

Few methods have been reported in literature for the determination of Pregabalin both in biological matrices [9-17] and pharmaceuticals [18-24] involving mass spectroscopy methods [9-15], pre-column derivatization methods [16-19], post-column derivatization methods [20], direct HPLC methods [21-23] and spectrophotometric, spectrofluorimetric methods [24]. To the best of our knowledge no method was reported for the determination of Pregabalin and its potential impurities in the bulk drug using UPLC for the regular analysis and stability studies in the quality control laboratory.

Ultra performance liquid chromatography (UPLC) is a new category of separation technique based upon well-established principles of liquid chromatography, which utilizes sub-2µm particles for stationary phase. These particles operate at elevated mobile phase linear velocities to affect dramatic increase in resolution, sensitivity and speed of analysis. Because of its speed and sensitivity, this technique is gaining considerable attention in recent years for pharmaceutical and biomedical analysis.

In the present work, this technology has been applied to the related substances and assay determination of Pregabalin drug substance. The objective of this research work was to develop a simple stability-indicating UPLC method for the related substances and assay determination of Pregabalin. The forced degradation was performed as per ICH recommended conditions, i.e acid, base and water hydrolysis, oxidative, thermal and photolytic stressed conditions to prove the stability-indicating ability of the method. The degradation impurities were identified using mass and MR techniques. The mixture of the degraded sample and its related impurities were used to optimize the method. The method was also validated as per ICH requirements [25]

Experimental:

Chemicals and Reagents:

Pregabalin and its related impurities were synthesized and purified using column liquid chromatography by the process research department of Active Pharmaceutical Ingredients, IPDO, Dr. Reddy's Laboratories (Hyderabad, India). The UPLC-grade acetonitrile and AR-grade dipotassium hydrogen phosphate, monopotassium dihydrogen phosphate, sodium hydroxide, hydrochloric acid and hydrogen peroxide which are required for the mobile phase preparation and degradation studies were purchased from Rankem (Mumbai, India). Millipore purified water (Milli-Q Plus; Bangalore, India) was used to prepare the mobile phase and wash solvents.

Instrumentation:

The method development attempts, forced degradation studies and subsequent validation of method were performed on Waters Acquity UPLC system with a diode array detector. The data were collected and processed using empower software. The photolytic degradation was carried out using Binder KBS240 photolytic chamber, New York, USA. The mass analysis of degradation products was performed on Agilent LCMS 6410 QqQ instrument, California, USA and Waters acquity HR-MS ultra performance liquid chromatography system coupled with a time of flight spectrometer. The NMR analysis of degradation products was performed on Varian 400MHz, oxford magnet, California, USA**.**

Chromatographic conditions:

The chromatographic separation was optimized on a Phenomenex kinetex phenyl-hexyl column with the dimensions of 100 mm x 2.1 mm and 1.7 µm as particle size. The gradient LC method employs water: monopotassium dihydrogen phosphate: dipotassium hydrogen phosphate in the ratio of 100:0.136:0.05 (*v/w/w)* as a mobile phase A and acetonitrile as mobile phase B. The UPLC gradient program was optimized as: time/% mobile phase B: 0/0, 7/50 and 10/0 with a post run time of 3 min. The flow rate of the mobile phase was 0.30 mL min^{-1}. The column temperature was maintained at 40°C and the detection wave length was set as 210 nm. The column loading was optimized as 10 µg of Pregabalin in 1µL injection volume. A mixture of water and acetonitrile in the ratio of 90:10 (*v/v*) was used as diluent.

Sample preparation:

Pregabalin solution was prepared at target analyte concentration (TAC), which is 10 mg mL^{-1} in the diluent for related substances and assay determination. The stock solutions of Imp-A

(alkene impurity), Imp-B (Diacid impurity), Imp-C (CMHA), Imp-D (Lactam impurity) and Imp-E (Dione impurity) were also prepared in the diluent for the preparation of system suitability solution with 0.15 % *w/w* (specification level) of each impurity at TAC of Pregabalin.

Method development and optimization:

The core objective of the chromatographic method is to get a sharp peak shape for Pregabalin, and to separate all potential impurities and the degradation products from the analyte in short run time, especially the critical pairs Pregabalin and Imp-B, and also Imp-D and Imp-E. The parameters such as affect of pH on analyte retention, type of buffer to use, and its concentration, solubility in the organic modifier and its affect on detection plays important role in reverse phase chromatography method development of ionic species.

An improper choice of buffer, in terms of buffering species, ionic strength and pH, can result in poor or irreproducible retention and tailing in reverse-phase separation of polar and ionizable compounds. The choice of buffer is also dependent upon means of detection. For traditional UV detection, the buffer needs to be selectively transparent in particular region, especially, critical for gradient separations. Phosphate buffers have low enough absorption at below 210 nm.

Considering the facts that the pKa value of Pregabalin is 4.2 for carboxylic group and 10.6 for amine group, pH value 7.4 – (which is neutral), more polar nature, and less UV active, it was focused to do the method development attempts with phosphate buffer. Phosphate buffers endearing qualities include its low cost, high purity, convenient preparation, useful pH ranges and good chromatographic behavior.

For Pregabalin, phosphate buffer given an excellent sharp peak shape with good resolution between all impurities. Initial attempts for the method development were made using variety of stationary phases which are listed in **Table 1**.

TABLE 1: METHOD DEVELOPMENT TRIALS AND OBSERVATIONS

Trials	Column	Dimension	Mobile phase	USP Tailing factor (*T*)
1	BEH-C8	50*2.1mm 1.7µ	Phosphate buffer with pH 6.2/Acetonitrile	2.9
2	BEH-C18	50*2.1mm 1.7µ	Phosphate buffer with pH 6.2/Acetonitrile	3.1
3	HSS-CN	50*2.1mm 1.8µ	Phosphate buffer with pH 6.2/Acetonitrile	3.0
4	HSS-CN	50*2.1mm 1.8µ	Ammonium acetate/Acetonitrile	3.1
5	BEH-PHENYL	50*2.1mm 1.7µ	Phosphate buffer with pH 6.2/Acetonitrile	2.3
6	BEH-PHENYL	50*2.1mm 1.7µ	Water/acetonitrile/trifluoroacetic acid	7.1

The tailing factor of the Pregabalin was observed more than 2.0 during the method development attempts on different stationary phases like C_8, C_{18}, cyano and phenyl with different selectivity using water / acetonitrile / trifluoroacetic acid, ammonium acetate and phosphate buffers as mobile phase. Stationary phase has played a significant role in achieving the good tailing factor of

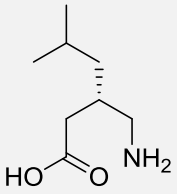
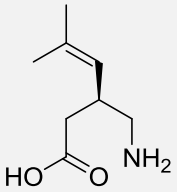
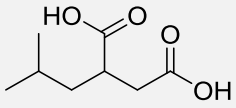
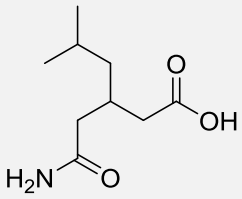
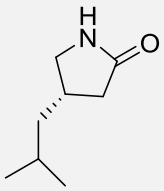
Pregabalin, and good separation between the critical pairs Pregabalin and Imp-B, and also Imp-D and Imp-E. Satisfactory peak shape and the resolution of closely eluting potential impurities were achieved on Phenomenex kinetex phenyl-hexyl column with the dimension of 100 mm x 2.1 mm and 1.7 µm as particle size, using solutions A and B as mobile phase.

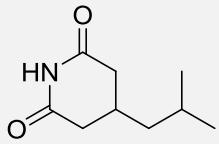
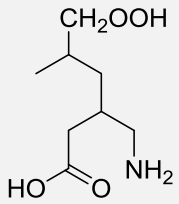
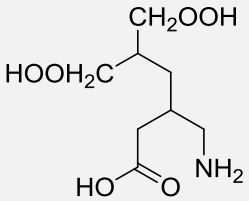
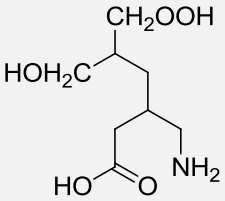
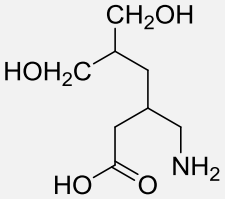
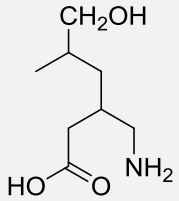
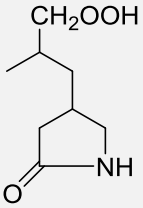
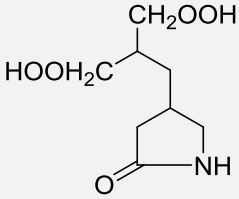
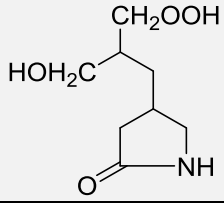
Kinetex Phenyl Hexyl columns[26] provide separations not achievable on C18 or C8 columns; such as increased retention for polar, aromatic compounds as well as reversals in analyte elution order. Most phenyl phases use a short propyl (3 carbon) linker, which limits phase stability. The Phenyl-Hexyl bonded phase employs a phenyl ring with a hexyl (6 carbon) linker and is densely bonded to Luna silica surface, reducing bonded phase hydrolysis and increasing chemical stability that results in highly reproducible and stable phenyl

phase, dual selectivity of both phenyl phase and a short alkyl phase (C5 or C8) gives excellent retention to polar amine compounds. Along with the advantages of the chemistry, Kinetex columns are core shell technology products from Phenomenex that provides increased efficiencies over traditional, fully porous columns. The precise architecture of core-shell particles provide dramatic leaps in the performance in two ways - high particle density that creates optimal bed structure which reduces band broadening effects of Eddy Diffusion and the thin, porous layer, or "shell", decreases the diffusion path length, thus reducing the time it takes for molecules to diffuse into and out of the particle. Because of all these advantages of kinetex phenyl-hexyl column, excellent peak shape and good resolution has been achieved for Pregabalin.

In the developed method mobile phase A was a mixture of water: monopotassium dihydrogen phosphate : dipotassium hydrogen phosphate in the ratio 100:0.136:0.05 (*v/w/w)* and mobile phase B was acetonitrile. The flow rate of the mobile phase was 0.30 mL min^{-1}. The UPLC gradient program was also played a vital role in the resolution of the Imp-B with the Pregabalin peak and in the resolution of Imp-D and Imp-E.

The UPLC gradient program was optimised as: time/% solution B: 0/0, 7/50 and 10/0 with a post run time of 3 min in order to get the better resolution. The column temperature was set as 40°C. The retention time of the Pregabalin with the optimized gradient program was 2.6 min which is appropriate, and the tailing factor of Pregabalin is found to be 1.0. In the optimized conditions it has been observed that the Pregabalin, Imp-A, Imp-B, Imp-C, Imp-D, Imp-E and the degradation impurities (**Fig. 1**) were well separated with a resolution greater than 4. The system suitability results are captured in **Table 2** and the developed UPLC method was found to be specific for Pregabalin, its known impurities and degradation impurities (**Fig. 2**).

Pregabalin		(S)-3-(aminomethyl)-5-methylhexanoic acid
Imp-A		(S)-3-(aminomethyl)-5-methylhex-4-enoic acid
Imp-B		3-Carboxy-5-methylhexanoic acid
Imp-C		3-(2-amino-2-oxoethyl)-5-methylhexanoic acid
Imp-D		(S)-4-isobutylpyrrolidin-2-one

Imp-E		4-isobutylpiperidine-2, 6-Dione
Imp-O1		3-(aminomethyl)-6-hydroperoxy-5-methylhexanoic acid
Imp-O2		3-(aminomethyl)-6-hydroperoxy-5-(hydroperoxymethyl)hexanoic acid
Imp-O3		3-(aminomethyl)-6-hydroperoxy-5-(hydroxymethyl)hexanoic acid
Imp-O4		3-(aminomethyl)-6-hydroxy-5-(hydroxymethyl)hexanoic acid
Imp-O5		3-(aminomethyl)-6-hydroxy-5-methylhexanoic acid
Imp-O6		4-(3-hydroperoxy-2-methylpropyl)pyrrolidin-2-one
Imp-O7		4-(3-hydroperoxy-2-(hydroperoxymethyl)propyl)pyrrolidin-2-one
Imp-O8		4-(3-hydroperoxy-2-(hydroxymethyl)propyl)pyrrolidin-2-one

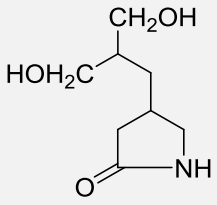
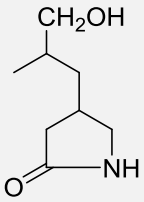
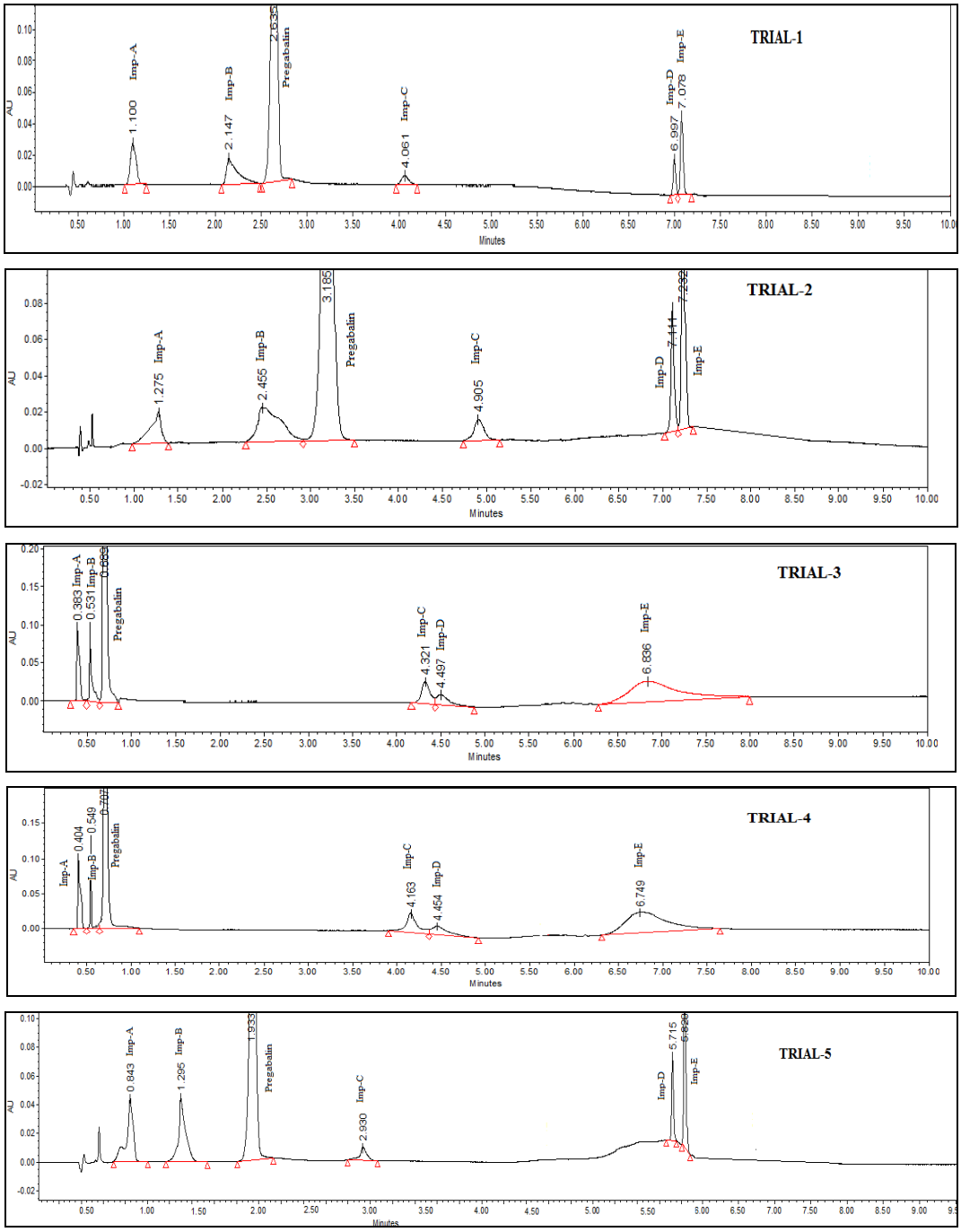
Imp-O9		4-(3-hydroxy-2-(hydroxymethyl)propyl)pyrrolidin-2-one
Imp-O10		4-(3-hydroxy-2-methylpropyl)pyrrolidin-2-one

FIG. 1: CHEMICAL STRUCTURE, NAME OF PREGABALIN AND ITS IMPURITIES



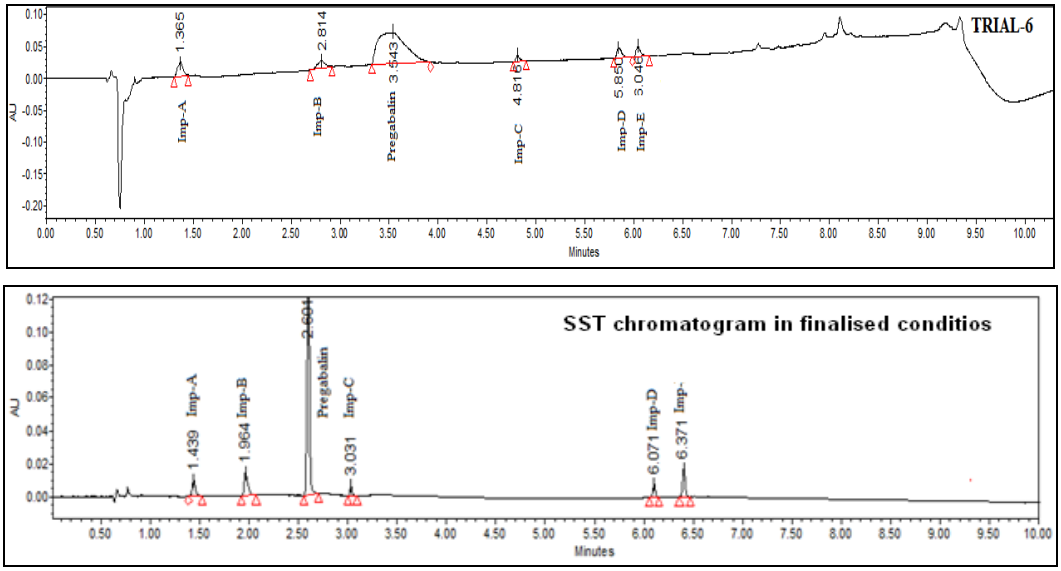


FIG. 2: TYPICAL CHROMATOGRAMS OF METHOD DEVELOPMENT TRIALS AND FINAL SST CHROMATOGRAM

TABLE 2: RESULTS OF SYSTEM SUITABILITY TEST

Compound ($n = 3$)	Retention time (*RT*)	Relative retention time (*RRT*)	Capacity factor (*k'*)	Resolution (R_s)	USP Tailing factor (*T*)	No. of Theoretical plates (*N*-Tangent method)
Imp-A	1.4	0.55±0.00	6.1±0.11	--	1.1±0.01	34757
Imp-B	2.0	0.76±0.01	8.8±0.02	7.4±0.16	1.2±0.01	71979
Pregabalin	2.6	1.00±0.00	12.0±0.11	10.4±0.14	1.0±0.00	88259
Imp-C	3.0	1.15±0.01	14.2±0.09	11.0±0.11	1.1±0.01	125827
Imp-D	6.0	2.33±0.00	29.4±0.07	10.7±0.09	1.0±0.00	295838
Imp-E	6.4	2.45±0.00	30.9±0.03	6.4±0.13	1.0±0.00	290929

Specificity:

Specificity is the ability of the method to measure the analyte in the presence of process related and the degradation impurities. The specificity of the developed UPLC method for Pregabalin was demonstrated in the presence of its known impurities, namely Imp-A, Imp-B, Imp-C, Imp-D and Imp-E and its degradation products. Thorough forced degradation studies were carried out on Pregabalin to ascertain the stability-indicating property of the developed method.

The stress conditions engaged for degradation studies as per the ICH preferred conditions includes photolytic, thermal, oxidation and hydrolysis with acid, base and water. The photolytic stressed studies were performed for 11 days as per ICH Q1B [27]. The thermal stress was done at 105°C for 10 days. The acid, base stress was performed with 1.0 N HCl and 1.0 N NaOH on Pregabalin for 5 days and 48 h respectively at ambient temperature (25±2°C). Water hydrolysis was performed for 5 days at ambient temperature. The oxidation stress was done with 10 % hydrogen peroxide for 24 h at ambient temperature [28-29]. All the stressed samples were quantified for Pregabalin and its impurities. Peak purity of stressed samples of Pregabalin and the spiked solutions of Imp-A, Imp-B, Imp-C, Imp-D and Imp-E were checked by waters acquity diode array detector (DAD). Additionally the unknown degradation products formed were identified by mass and NMR techniques.

Method validation:

Precision:

Precision is the closeness of agreement between a series of measurements obtained from multiple sampling of same sample under the prescribed conditions. Six individual measures of Pregabalin were performed with 0.15 % *w/w* of each Imp-A, Imp-B, Imp-C, Imp-D and Imp-E to the reference of TAC. Quantification of individual impurities and Pregabalin was performed for each of the preparations and the percent relative standard

deviation (RSD) was determined for the content of the impurities and the assay. To evaluate the intermediate precision, the same experiment was repeated with a different lot of column and a different instrument in the same laboratory.

Limit of detection (LOD) and limit of quantification (LOQ):

The limit of detection (LOD) and limit of quantification (LOQ) of an individual analytical procedure are the lowest amounts of analyte in a sample that can be detected and quantitatively determined with suitable precision and accuracy respectively. The LOD and LOQ for each of the impurities were established by attaining signal-to-noise ratio of approximately 3:1 and 10:1 respectively, from a series of dilute solutions with known concentrations. Precision was carried out at LOQ level by preparing six individual preparations of Pregabalin with its related impurities at LOQ level and calculating the percentage RSD for the areas of Pregabalin and its related impurities. Accuracy at LOQ level was also carried out by preparing three recovery solutions of Pregabalin with its related impurities at LOQ level and calculating the percentage recovery for areas of all related impurities.

Linearity:

The linearity of an analytical procedure is its ability to obtain test results that are directly proportional to the amount of analyte in the sample. The linearity of method was demonstrated separately at impurity level and assay level. The solutions of Pregabalin with its known impurities were prepared at five different concentrations from LOQ to 0.30 % *w/w* (LOQ, 0.05, 0.10, 0.15, 0.20 and 0.30 % *w/w*) of TAC for the linearity at impurity level. The assay linearity was performed by preparing five different solid weighing of Pregabalin from 80 % to 120 % *w/w* (80, 90, 100, 110 and 120 % *w/w*) with respect TAC and injected.

Using least-squares analysis, the regression line was plotted with area versus concentration. The value of the slope, *Y*-intercept and % *Y*-intercept of the calibration curves were calculated. The relative response factor (RRF) of each impurity was determined by dividing the slope of the each impurity with slope of Pregabalin.

Accuracy:

The accuracy of an analytical procedure expresses the closeness of agreement between the values determined by the method and conventional true value or an accepted reference value. Accuracy of impurities at each level was established by standard addition of the known quantities of impurities in test sample and calculation of the recovery. The study was carried out in triplicate at 0.075, 0.15 and 0.225 % *w/w* of the TAC.

The percentage of recoveries of Imp-A, Imp-B, Imp-C, Imp-D and Imp-E were calculated from the original quantity spiked and the amount of the same calculated against the main peak diluted to impurity specification level with RRF correction. The accuracy of the assay was evaluated in triplicate at three concentration levels, i.e. 8, 10 and 12 mg mL-1 of Pregabalin, corresponding to 80, 100 and 120 % *w/w* of the TAC. The percentage recovery at each level was calculated against the Pregabalin standard, considered 99.1 % *w/w* as the true value derived by the mass balance approach.

Robustness:

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters. Deliberate changes were made from original experimental conditions to record the tailing factor of the Pregabalin and the resolution between Pregabalin, Imp-A, Imp-B, Imp-C, Imp-D and Imp-E to determine the robustness of the developed method.

The effect of the flow rate was studied at 0.25 mL min^{-1} and 0.35 mL min^{-1}, instead of 0.30 mL min^{-1}. The effect of wave length was studied at 208 nm and 212 nm, instead of 210 nm. The effect of the column temperature was studied at 35°C and 45°C, instead of 40°C. The effect of the gradient program was studied with program time/% mobile phase B: 0/0, 7/45 and 0/0, 7/55, instead of 0/0, 7/50.

Solution stability and mobile phase stability:

The solution stability and mobile phase stability provide an indication of the method's reliability in normal usage during the storage of the solutions used in the method. The solution stability of Pregabalin was studied for 48h at room

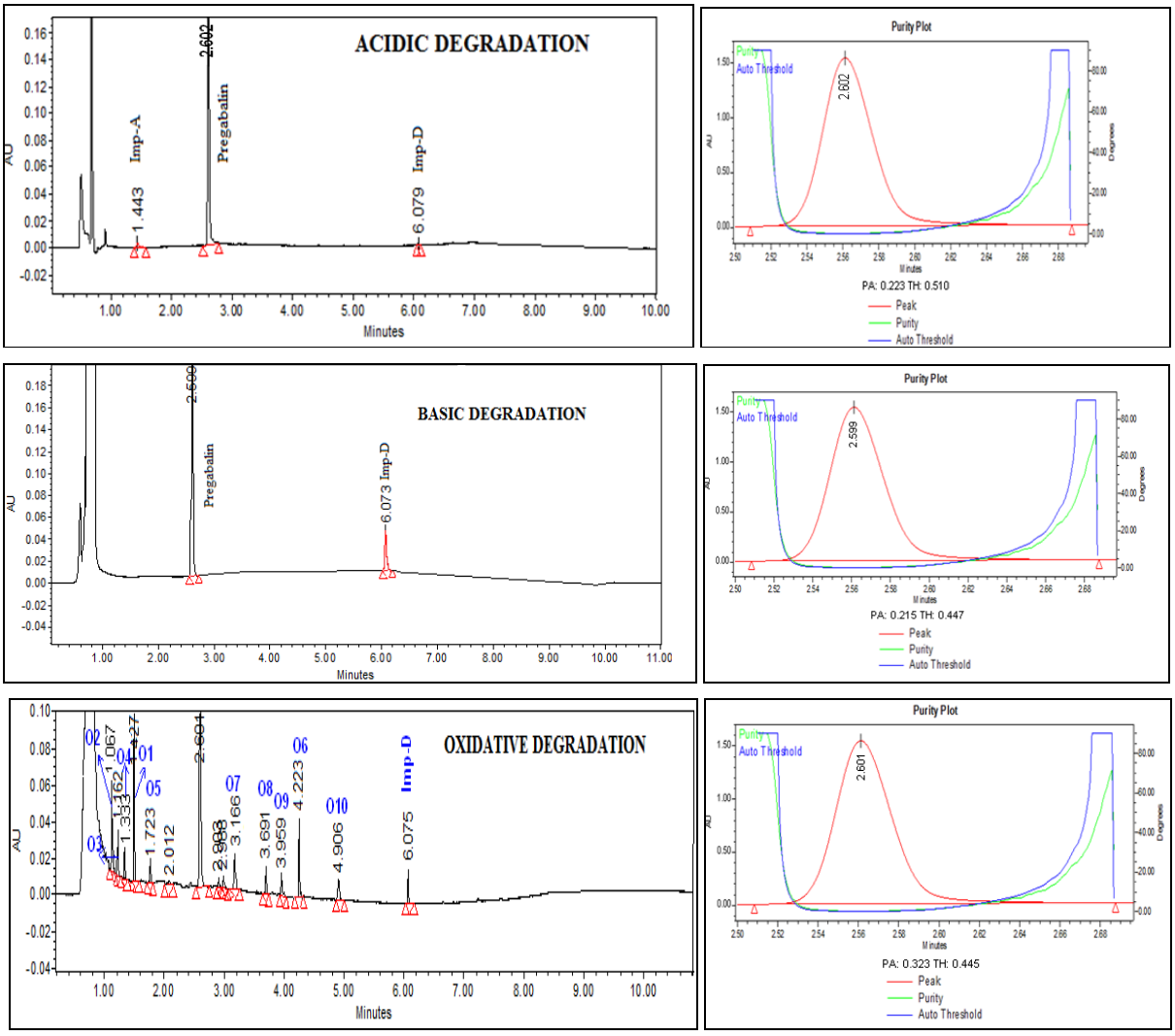
temperature. The reference standard of Pregabalin and the sample spiked with impurities at specification level were injected every 6h. The content of impurities and Pregabalin were quantified at each interval up to the study period. The mobile phase stability was also established by quantifying the freshly prepared sample solutions against freshly prepared reference standard solutions every 6h. During the study period, the prepared mobile phase remained unchanged. The recovery of the Pregabalin assay and the content of each impurity were calculated against the initial value of the study period.

RESULTS AND DISCUSSION:

Results of forced degradation studies:

The degradation of drug substance was not observed in water hydrolysis and photolytic stress conditions. Slight degradation of drug substance was observed in acidic hydrolysis and thermal stress conditions, and significant degradation of drug substance was observed in base hydrolysis and oxidative stress conditions. Pregabalin under acidic hydrolysis, base hydrolysis and thermal stress conditions leaded to the formation of known impurity-D. The drug substance Pregabalin under oxidative stress conditions leaded to the formation of ten unknown degradation impurities O1 to O10 along with known impurity-D.

The peak purity factor was within the threshold limit for all stressed samples, which demonstrates the specificity of the Pregabalin peak (**Fig. 3**). The assay of Pregabalin is unaffected in the presence of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E and its degradation products, the mass balance of stressed samples was between 98.0 and 101.0 % *w/w* when the RRF of the degradant was considered to be one, which confirms the specificity and stability indicating ability of the developed method. The synopsis of the forced degradation was captured in **Table 3** and **4**.



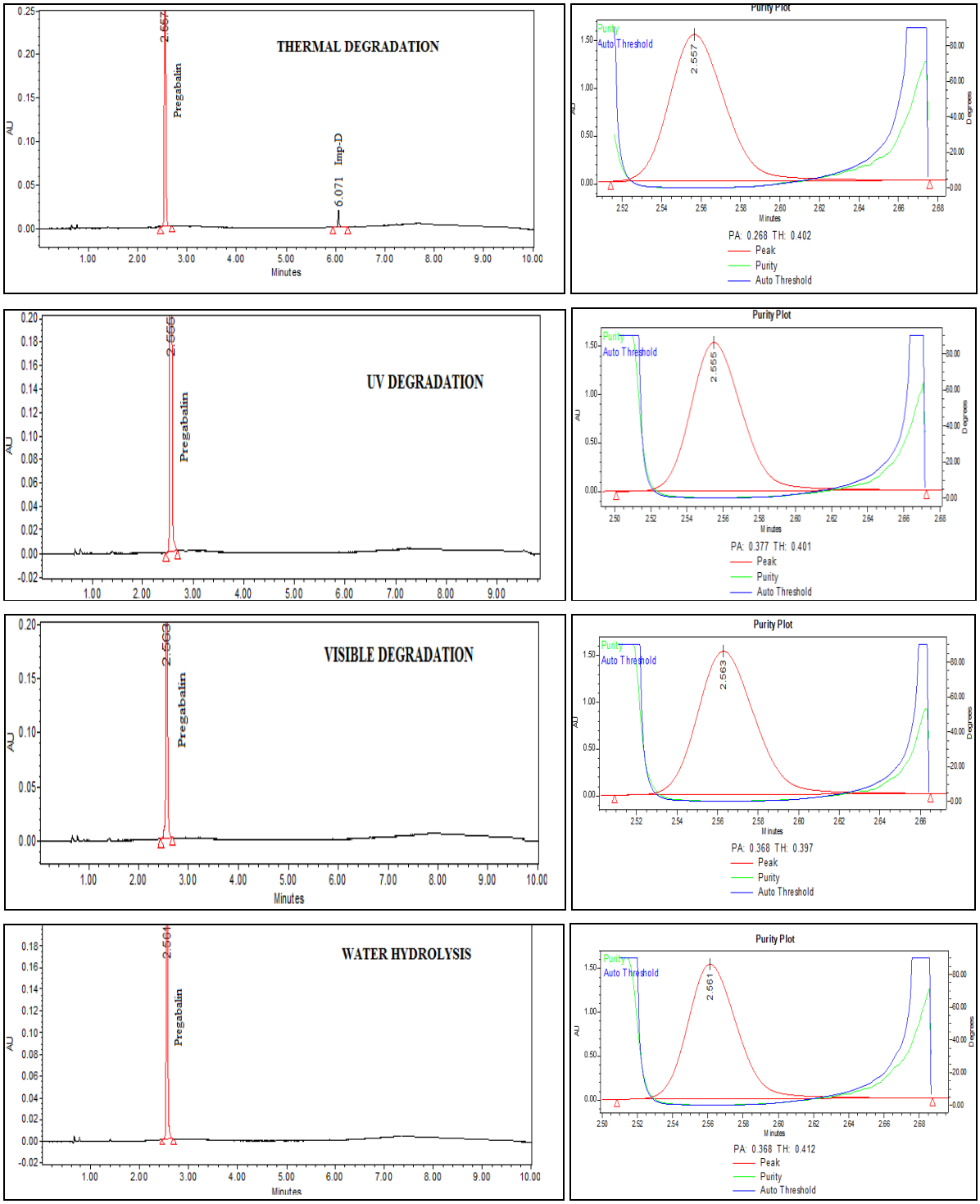


FIG. 3: TYPICAL CHROMATOGRAMS OF SPECIFICITY

TABLE 3: SUMMARY OF FORCED DEGRADATION RESULTS

Stress condition	Duration	Purity of Analyte after degradation	Assay of Analyte after degradation	Observations
Unstressed sample	--	99.9	99.8	--
Water hydrolysis	5 days	99.8	99.6	No degradation products formed
Acid hydrolysis (1N HCl)	5 days	99.1	99.2	Slight degradation was observed. Known Impurities A & D were formed
Base hydrolysis (1N NaOH)	48 hours	94.1	94.3	Significant degradation was observed. Known Impurity-D was formed
Oxidation (10% H2 O2)	72 hours	80.9	80.6	Significant degradation was observed. Unknown degradation impurities-O1 to

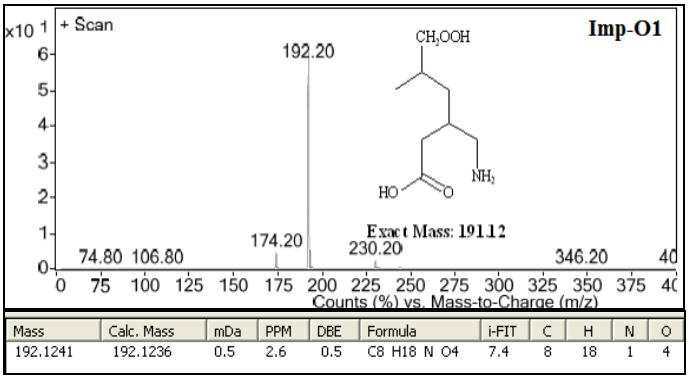
				O10 along with known Impurity-D were formed
Thermal (105° C)	10 days	97.9	97.8	Slight degradation was observed. Known Imp-D was formed
Photolytic degradation as per ICH guidelines both in UV & Visible	11 days	99.8	99.8	No degradation products formed

TABLE 4: DETAILS OF DEGRADATION PRODUCTS

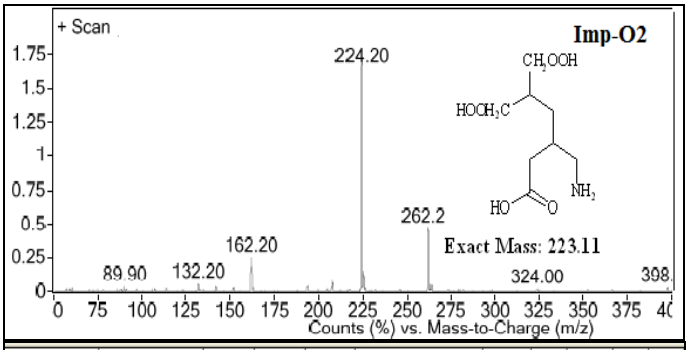
Degradation Impurity	Mass number (m/z)	Relative retention time (*RRT*)	USP Tailing factor *(T)*
O2	224	0.41	1.0
O3	208	0.45	1.0
O4	192	0.55	1.0
O1	192	0.66	1.0
O5	176	0.78	1.0
O7	206	1.22	1.1
O8	190	1.42	1.0
O9	174	1.52	1.0

Oxidative degradation pathways of Pregabalin and identification of degradation products:

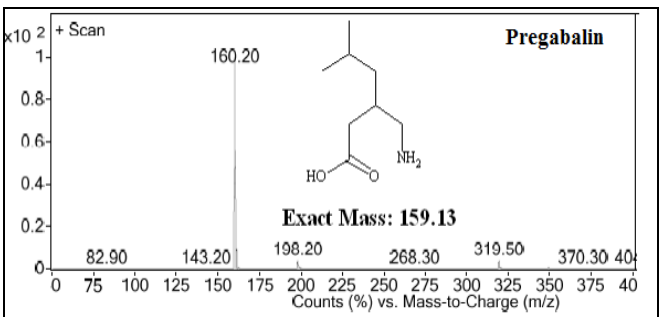
The oxidative degradation pathways of Pregabalin were studied by subjecting the drug substance to oxidation with 10% hydrogen peroxide for 7 days. The oxidation of Pregabalin leaded to the formation of ten unknown degradation impurities O1 to O10 along with known impurity-D. A LC–MS study was carried out to determine the m/z values of the major degradation products using an Agilent 1100 series liquid chromatography system coupled with a 6410 series triple quadruple mass spectrometer. A HR-MS study was done to determine the molecular formulas of the degradation products using Waters acquity ultra performance liquid chromatography system coupled with a time of flight spectrometer. The oxidative degradation sample was subjected to ^{1}HNMR, ^{13}CNMR, COSEY and HSQC to elucidate the structures of unknown degradation impurities. Based on the above studies the proposed structures for the degradation impurities are shown in **Fig. 1**, and the typical mass spectra, HR-MS and NMR data are shown in **Fig 4**.



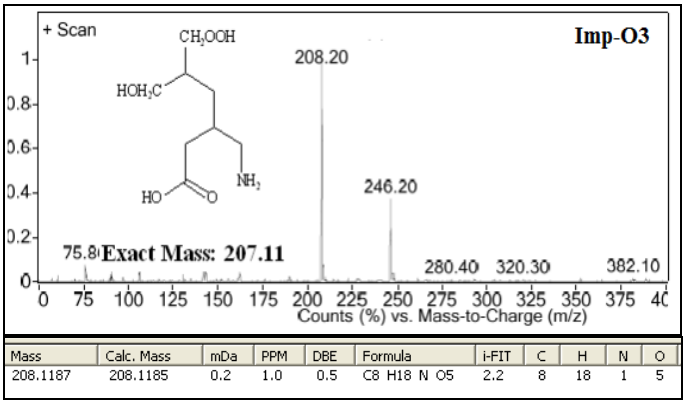
Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	C	H	N	O
192.1241	192.1236	0.5	2.6	0.5	C8 H18 N O4	7.4	8	18	1	4



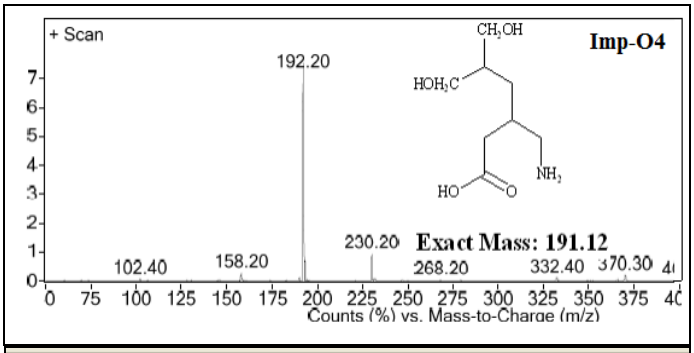
Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	C	H	N	O
224.1147	224.1134	1.3	5.8	0.5	C8 H18 N O6	2.5	8	18	1	6



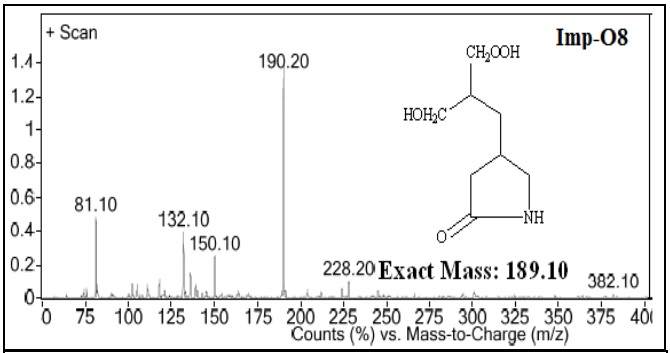
Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	C	H	N	O
160.1339	160.1338	0.1	0.6	0.5	C8 H18 N O2	31.2	8	18	1	2



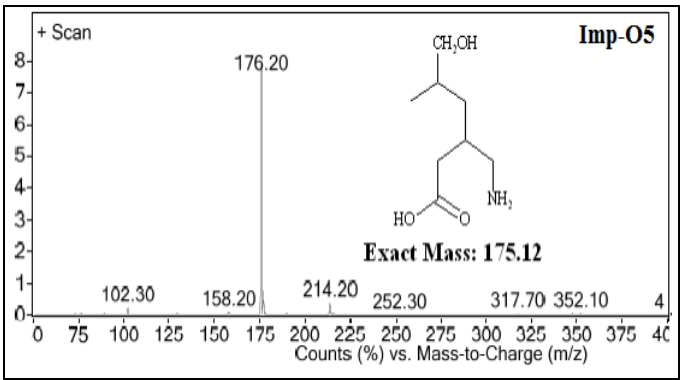
Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	C	H	N	O
208.1187	208.1185	0.2	1.0	0.5	C8 H18 N O5	2.2	8	18	1	5



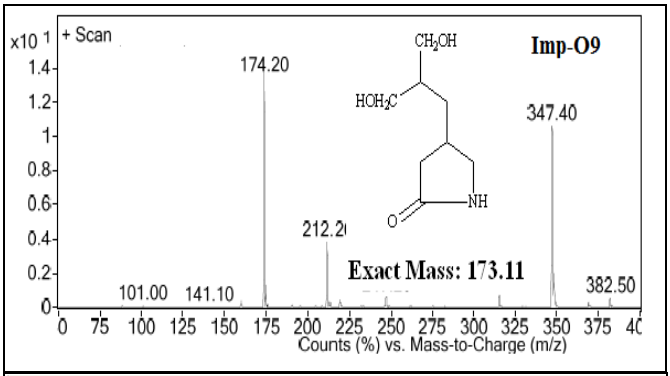
Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	C	H	N	O
192.1239	192.1236	0.3	1.6	0.5	C8 H18 N O4	78.0	8	18	1	4



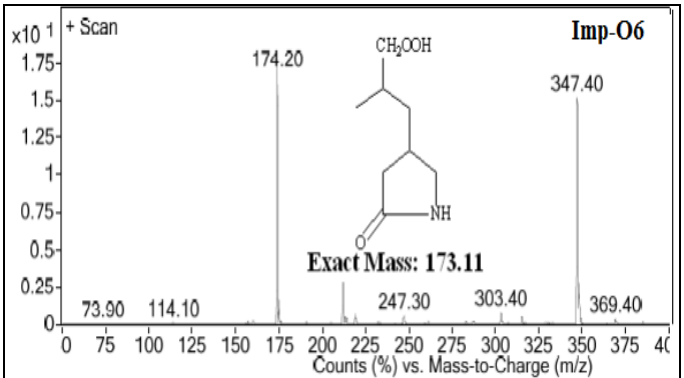
Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	C	H	N	O
190.1697	190.1689	0.8	4.2	1.5	C8 H16 N O4	16.8	8	16	1	4



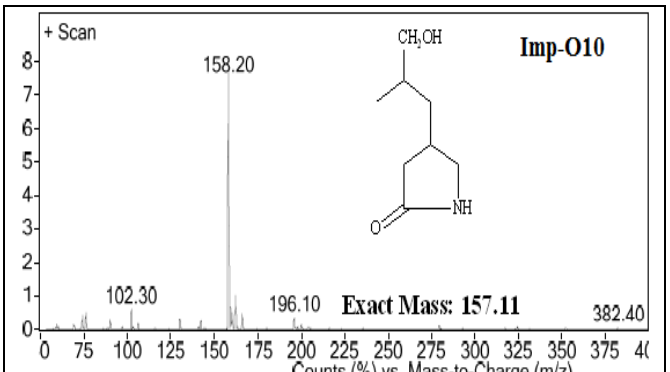
Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	C	H	N	O
176.1290	176.1287	0.3	1.7	0.5	C8 H18 N O3	236.5	8	18	1	3



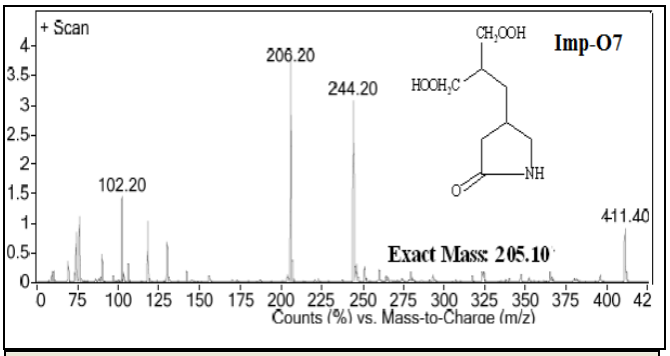
Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	C	H	N	O
174.1150	174.1142	0.8	4.6	1.5	C8 H16 N O3	15.9	8	16	1	3



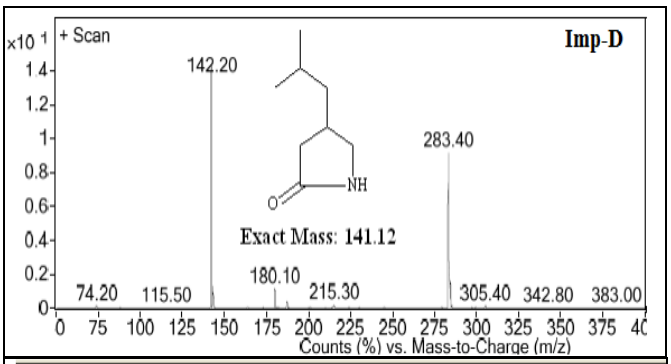
Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	C	H	N	O
174.1140	174.1130	1.0	5.7	1.5	C8 H16 N O3	945.5	8	16	1	3



Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	C	H	N	O
158.1199	158.1192	0.7	4.4	1.5	C8 H16 N O2	14.4	8	16	1	2



Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	C	H	N	O
206.1038	206.1028	1.0	4.9	1.5	C8 H16 N O5	4.9	8	16	1	5



Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	C	H	N	O
142.1246	142.1238	0.8	5.6	1.5	C8 H16 N O3	945.5	8	16	1	3

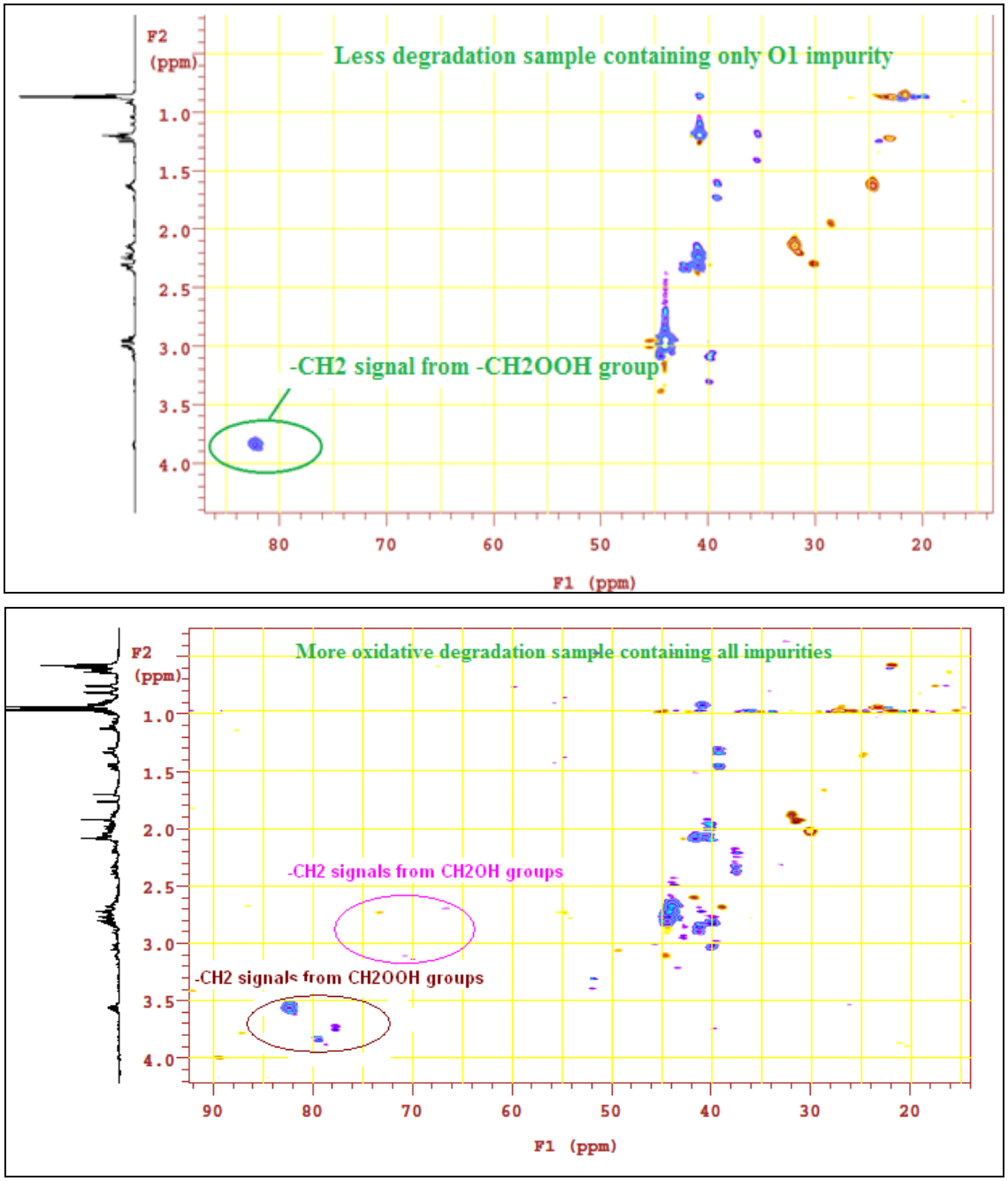


FIG. 4: TYPICAL LC-MS, HR-MS AND NMR DATA OF DEGRADATION PRODUCTS

Pregabalin on oxidation with hydrogen peroxide leaded to the continuous formation of mono hydroperoxide (191.1) as major degradant [30-31] and lactum impurity (141.1) as minor degradant. The mono hydroperoxide was further converted to cyclized compound of mono hydroperoxide (173.1), dihydroperoxide (223.1) and mono hydroxy (175.1) compounds, these impurities further converted into the remaining impurities and the detailed degradation pathways were presented in **Fig. 5**

Precision: All individual values of impurity content and the assay in the precision and intermediate precision studies fall well within the range of the average confidence interval, confirming the excellent precision of the method. The recommended precision values in terms of percentage RSD should be not more than 15 for the related substances and not more than 2.0 for the assay. However, the percentage RSD of the content of impurities and the assay of Pregabalin in the precision study, including intermediate precision, were well within 3.9 and 0.41, respectively. The percentage RSD values are reported in **Table 5** and **6** for related impurities and Pregabalin respectively.

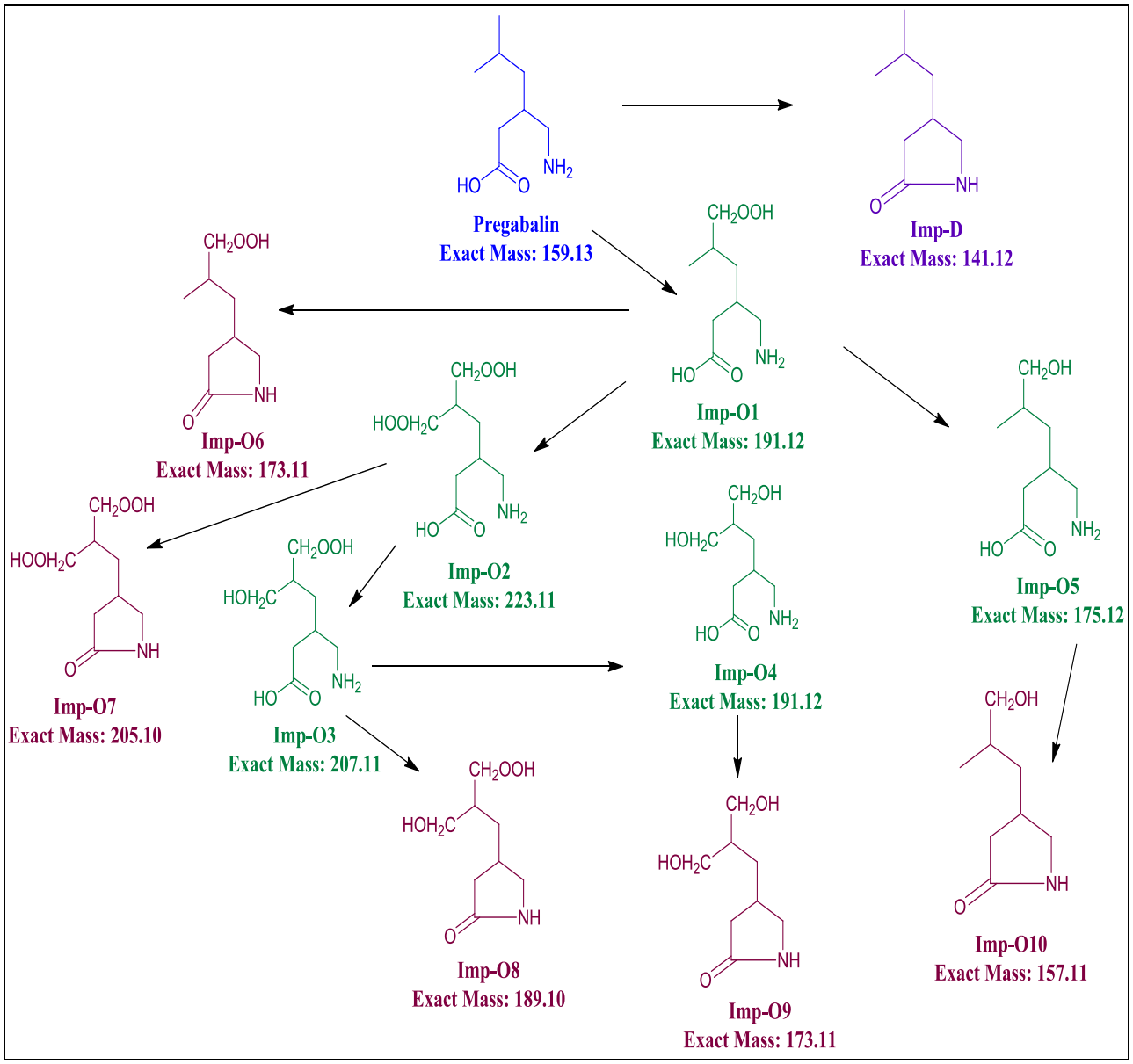


FIG 5: PROBABLE DEGRADATION PATHWAYS

TABLE 5: RESULTS OF VALIDATION PARAMETERS FOR RELATED IMPURITIES

Parameter	Pregabalin	Imp-A	Imp-B	Imp-C	Imp-D	Imp-E
LOD (% *w/w* with respect to TAC)	0.01	0.02	0.02	0.02	0.01	0.01
LOD (µg mL^{-1})	1.4	2.0	1.8	1.9	1.2	1.1
LOQ (% *w/w* with respect to TAC)	0.04	0.06	0.05	0.05	0.03	0.03
LOQ (µg mL^{-1})	4.1	5.9	5.2	5.7	3.4	3.3
Linearity						
Slope (*m*)	374781	419755	397268	416007	367285	423578
Intercept (*C*)	1314	1391	642	-941	-483	1409
% *Y*-intercept	0.15	1.21	1.45	-2.3	-1.07	0.57
Correlation coefficient	0.9996	0.9991	0.9993	0.9991	0.9998	0.9999
Precision at LOQ level (%RSD for *n* = 6)	1.17	0.77	1.46	1.06	0.32	1.09
Precision (%RSD for *n* = 6)	--	2.17	2.77	1.56	3.17	0.91
Ruggedness(%RSD for *n* = 6)	--	1.34	3.12	2.05	3.86	0.87
Relative response factor	1.00	1.12	1.06	1.11	0.98	1.13

Table 6: RESULTS OF VALIDATION FOR PREGABALIN AT ASSAY LEVEL

Parameter	Pregabalin
Linearity	
Slope (*m*)	39842312
Intercept (*C*)	135184
% *Y*-intercept	0.21
Correlation coefficient	0.9999
Precision (%RSD for *n = 6*)	0.32
Ruggedness(%RSD for *n = 6*)	0.41
% Recovery for *n = 3*	
80% level	99.5 ± 0.15
100% level	99.9 ± 0.17
120% level	100.2 ± 0.27

Limit of detection and limit of quantification:

The limit of detection of Pregabalin, Imp-A, Imp-B, Imp-C, Imp-D and Imp-E were less than or equal to 0.02 % *w/w* (of TAC) for 1µL injection volume. The limit of quantification of Pregabalin, Imp-A, Imp-B, Imp-C, Imp-D and Imp-E were less than or equal to 0.06 % *w/w* (of TAC) for 1µL injection volume. The percentage RSD of impurities at LOQ level were less than 1.5 and the recovery values at LOQ level were between 94.7 and 98.8. Since the dosage of the Pregabalin was less than 150-300mg per day, the limit of quantification at the reporting threshold for the known impurities and the API holds good for the necessity of the method.

These limits of quantification levels of the impurities were helpful for the process research work to control the impurities at the accepted level during the optimization of the process. The LOD & LOQ values of Pregabalin and its related impurities, and precision at LOQ level are tabulated in **Table 5**. The results of accuracy at LOQ level are tabulated in **Table 7**.

TABLE 7: ACCURACY FOR RELATED SUBSTANCES

Amount spiked	% Recovery for *n = 3*				
	Imp-A	Imp-B	Imp-C	Imp-D	Imp-E
LOQ	97.1± 0.11	98.4± 0.37	97.5± 0.37	97.4± 0.11	95.3± 0.57
0.075 % *w/w* of TAC	97.8± 0.78	97.8± 0.68	96.4± 0.52	97.6± 0.32	96.8± 0.59
0.15 % *w/w* of TAC	98.4± 0.13	99.4± 0.25	98.5± 0.57	100.1± 0.14	100.7± 0.57
0.225 % *w/w* of TAC	100.2± 0.57	99.7± 0.24	98.6± 0.54	100.3± 0.24	100.2± 0.36

Linearity:

Excellent correlation was achieved for the regression line of Pregabalin and its related impurities at LOQ to 200 % of the specification level. The correlation coefficient obtained for all the plots was greater than 0.999. The RRF of each impurity was very close to Pregabalin for all impurities at the optimized condition. The *Y*-intercept of each plot was below 2.3 % of the response at 0.15 % *w/w* level of the corresponding impurity. This indicates that the achieved RRF value is nearer to the true value because the plots almost go through the origin.

Linear calibration plot for the assay was obtained over the calibration ranges tested, i.e., 8 to 12 mg mL^{-1}. An excellent correlation was obtained between the peak area and concentration of Pregabalin by achieving a correlation coefficient greater than 0.999. The *Y*-intercept for the assay concentration also supports that the plot goes almost through the origin. The linearity results and RRF values are tabulated in **Table 5**.

Accuracy:

The percentage recovery of each impurity falls in the range of 95.9 to 101.3 **Table 7**. The individual assay value at each level in triplicate is close to the derived true value **Table 6**. All individual recovery values of the assay and impurities fell well within the confidence interval of mean values. Good recovery values reflecting the exact values of RRF of impurities as well as the capability of accuracy of the method.

Robustness:

In all the deliberate varied chromatographic conditions i.e. flow rate, wave length, column temperature and mobile phase ratio by gradient change, the tailing factor of the Pregabalin was less than 1.1 and the resolution for the critical pair Pregabalin and Imp-B was greater than 6.2, and for the critical pair Imp-D and Imp-E was greater than 4.5. There was a very minor variation in the resolution and tailing factor results observed in all the robustness conditions illustrating the robustness of the method. Though the higher column temperature shows better system suitability

parameters comparatively, it is preferable to run in nominal temperature when considering the durability of the column. The results are tabulated in **Table 8**.

TABLE 8: RESULTS OF ROBUSTNESS PARAMETERS

Parameter	Actual value	Changed value	No. of Theoretical plates (*N*-Tangent method)	USP Tailing factor (*T*)	Resolution (R_s) between Pregabalin and Imp-B	Resolution (R_s) between Imp-D and Imp-E
Flow rate	0.3 mL min^{-1}	0.27 mL min^{-1}	60123	1.1	9.2	6.7
		0.33 mL min^{-1}	95148	1.0	6.2	4.5
Wave length	210 nm	208 nm	87541	1.0	9.2	6.3
		212 nm	88459	1.0	9.3	6.3
Temperature	40°C	35°C	81698	1.1	9.1	5.7
		45°C	102564	1.0	12.3	7.9
Time/% mobile phase B	0/0,7/50	0/0, 7/45	80547	1.1	9.8	7.1
		0/0, 7/55	89264	1.0	6.4	4.9

Solution stability and mobile phase stability:
The percentage RSD of the assay of Pregabalin during solution stability and mobile phase stability experiments was within 1.0. No significant changes were experienced in the content of any of the impurities during solution stability and mobile phase stability experiments. The percentage recovery of the assay at each time point against the initial value was between 99.4 and 100.6. The percentage recovery of the content of each impurity against the initial value was between 97.1 and 102.4.

The solution stability and mobile phase stability experiment data confirm that the mobile phase and sample solutions were stable up to 48 h. This helps to reduce the time consumption of analysis and number of samples can be analysed till 48 hours in the same sequence in the quality control during regular analysis.

CONCLUSIONS: The developed simple UPLC method for related substance and assay determination of Pregabalin is linear, precise, accurate and specific. The short run time of the developed method significantly saves lot of analysis time (~6 times faster) as well as the solvents cost (~3 times lesser). The results of the validation carried out for the method satisfied the ICH requirements. This method can be used for the detection and quantification of known, unknown and degradation impurities in the Pregabalin drug substance during routine analysis and also for stability studies in view of its capability to separate degradation products.

Communication number **IPDO IPM - 00275** has been allotted for this research article in the research laboratory.

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