Research Article [Vukkum *et al.*, 5(11): Nov., 2014:3986-4006]
CODEN (USA): IJPLCP ISSN: 0976-7126

INTERNATIONAL JOURNAL OF PHARMACY & LIFE SCIENCES
(Int. J. of Pharm. Life Sci.)

Isolation and structural characterization of Imipramine hydrochloride degradation impurities and development of stability-indicating UPLC method

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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 Imipramine 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. During forced degradation it has been observed significant degradation of drug substance in acid hydrolysis and oxidative conditions. Two of the major degradation impurities are isolated using semi preparative HPLC, and the structures are elucidated using ^{1}HNMR, ^{13}CNMR, 2D NMR (COESY, HSQC, HMBC) and mass spectral data. Based on the complete spectral analysis, these two degradation impurities are designated as 10-(3-(dimethylamino)propyl)acridin-9(10H)-one (acid hydrolysis) and 3-(10,11-dihydro-5H-dibenzo[b,f]azepin-5-yl)-N,N-dimethylpropan-1-amineoxide (oxidative degradation). The separation of known impurities and degradation impurities are accomplished using a YMC TriArt C_{18} stationary phase with 100 mm length and 1.9 µm particle size in short run time (10 min). The developed method employs a linear gradient elution with ammonium acetate buffer, and mixture of acetonitrile and methanol as mobile phase, and is validated in accordance with International Conference on Harmonization requirements.

Key-Words: Imipramine, Ultra performance liquid chromatography (UPLC), Stability-indicating methods, Structural characterization of degradation impurities, Nuclear magnetic resonance spectroscopy & High resolution mass spectrometer

Introduction

Imipramine hydrochloride, the original tricyclic antidepressant, is a member of the dibenzazepine group of compounds. It is chemically known as 5-3-(Dimethylamino)propyl-10,11-dihydro-5*H*-dibenz[*b*,*f*]-azepine monohydrochloride (Fig 1). Imipramine is commonly used in the patients with depression; abnormal levels of chemicals in the brain (called neurotransmitters) may be the cause of their depression. These neurotransmitters are chemicals that the nerves in the brain use to communicate with each other. Imipramine is believed to elevate mood of patients by interfering to the reuptake of norepinephrine or serotonin [1].

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Imipramine hydrochloride is a prescription drug sold under the trade name Tofranil and the maximum recommended dose is 75 mg to 300 mg a day. Few methods have been reported in literature for the determination of Imipramine both in biological matrices and pharmaceuticals, involving European pharmacopeia method [2], HPLC [3–12], TLC [13], GC [14–17], LC with direct injection and electrochemical detection [18], adsorptive stripping voltammetry [19], chemometric methods [20], flow-injection extraction spectrophotometry [21], derivative spectrophotometry [22-23], and visible spectrophotometry [24–27]. Most of the reported methods for the determination of Imipramine suffer from one or the other disadvantages like time consuming and require expensive experimental setup [14–17], and chemometric methods are less sensitive [20]. And also the reported spectrophotometric methods are less sensitive [21–27] and require extraction procedures [26] and costly chromogenic reagent [27]. Considering these demerits, there is a

need to develop a more advantageous chromatographic method for the determination of Imipramine, its related impurities and degradation impurities.

To the best of our knowledge no UPLC method was reported in literature with detailed forced degradation studies on Imipramine, isolation and structural characterization of degradation impurities, and the determination of Imipramine and its potential impurities in short run time. Ultra performance liquid chromatography (UPLC) is a new category of separation technique based upon well-established principles of liquid chromatography, which utilizes sub-2 µm particles for stationary phase. These particles operate at elevated mobile phase linear velocities to affect dramatic increase in resolution, sensitivity and speed of analysis [28]. Because of its speed and sensitivity, this technique is gaining considerable attention in recent years for pharmaceutical and biomedical analysis [29-32].

In the present work, this technology has been applied to the related substances and assay determination of Imipramine 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 Imipramine, and the isolation and structural characterization of degradation impurities. 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 ^{1}H, ^{13}CNMR, two dimensional (2D) NMR (COESY, HSQC, HMBC), FT-IR and HR-MS data. 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 [33].

Material and Methods

Chemicals and Reagents

Imipramine hydrochloride and its related impurities were synthesized and purified using column liquid chromatography by the process research department of custom pharmaceutical services, Dr. Reddy's Laboratories (Hyderabad, India). The UPLC-grade acetonitrile, methanol and AR-grade ammonium acetate, 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.

Ultra performance liquid chromatography

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 chromatographic separation was optimized on a YMC TriArt C_{18} column with the dimensions of 100 mm x 2.0 mm and 1.9 µm as particle size. The gradient LC method employs 20 mM ammonium acetate as mobile phase A, and acetonitrile: methanol in the ratio of 50:50 (*v/v*) as mobile phase B. The UPLC gradient program was optimized as: time/% mobile phase B: 0/40, 10/90 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 254 nm. The column loading was optimized as 1 µg of Imipramine in 1 µL injection volume. A mixture of water and acetonitrile in the ratio of 50:50 (*v/v*) was used as diluent.

Semi preparative high pressure liquid chromatography

The isolation of degradation impurities were performed on semi preparative HPLC, make: Agilent 1100 series, 100 mL min^{-1} pump capacity with a diode array detector. The data were collected and processed using chemstation software. The separation was optimized on a YMC TriArt C_{18} column with the dimensions of 250 mm x 20 mm ID. The gradient method employs 20 mM ammonium acetate as mobile phase A, and acetonitrile: methanol in the ratio of 50:50 (*v/v*) as mobile phase B. The gradient program was optimized as: time/% mobile phase B: 0/20, 20/90. The flow rate of the mobile phase was 19 mL min-1.

NMR spectroscopy

The ^{1}H and two dimensional (2D) NMR (COESY, HSQC, HMBC) measurements were performed on Varian Mercury plus 400 MHz NMR instrument at 25°C in DMSO-d_6. ^{13}C NMR experiments were performed on a Varian Mercury 100 MHz instrument, model 2000 at 25°C in DMSO-d_6. The chemical shift values were reported on the δ scale in ppm, relative to TMS (δ = 0.00 ppm) and DMSO-d_6 (δ = 39.5 ppm) as internal standards, respectively. Assignments were further confirmed by running two-dimensional chemical shift correlation experiments.

FT-IR spectroscopy

FT-IR spectra for degradation impurities were recorded in the solid state as KBr dispersion using a Perkin Elmer spectrum one spectrophotometer.

Mass spectroscopy

Mass spectra were recorded on a Waters acquity HR-MS ultra performance liquid chromatography system coupled with a time of flight spectrometer. Detection of ions was performed in electrospray ionization - positive ion mode.

Sample preparation

Imipramine solution was prepared at target analyte concentration (TAC), which is 1 mg mL^{-1} in the diluent for related substances and assay determination. The stock solutions of DG1(Acid degradation impurity), Depramine, Desipramine, DG2 (oxidative degradation impurity), Imp-A and Iminodibenzyl 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 Imipramine.

Method development and optimization

The core objective of the chromatographic method was to get a sharp peak shape for Imipramine, and to separate all potential impurities and the degradation products from the analyte in short run time, especially to separate the impurities DG2 and Imp-A from Imipramine. Considering the facts that the p*Ka* value of Imipramine was 9.2 and it was highly basic, it was focused to do the method development attempts in neutral & basic conditions. Initial attempts for the method development were made using variety of stationary phases. The tailing factor of the Imipramine 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. Ammonium acetate buffer given an excellent sharp peak for Imipramine, with good resolution between all impurities. Stationary phase has played a significant role in achieving the good tailing factor of Imipramine, and good separation between the DG2 and Imp-A from Imipramine. Satisfactory peak shape and the resolution of closely eluting potential impurities were achieved on YMC TriArt C_{18} column with the dimension of 100 mm x 2.0 mm and 1.9 µm as particle size, using solutions A and B as mobile phase. The YMC TriArt C_{18} columns are conventional hybrid silica-based ODS columns tend to be less hydrophobic than silica based columns. YMC TriArt C_{18} [34] has a higher carbon load, making its hydrophobicity comparable to standard ODS columns, making it a versatile first choice column for method development.

In the developed UPLC method mobile phase A was 20 mM ammonium acetate, and mobile phase B was acetonitrile: methanol in the ratio 50:50 (*v/v*). 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 DG2 and Imp-A peaks from Imipramine peak. The UPLC gradient program was optimised as: time/% solution B: 0/40, 10/90 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 Imipramine with the optimized gradient program was 5.3 min which is appropriate, and the tailing factor of Imipramine is found to be 1.2. In the optimized conditions it has been observed that the Imipramine, Deparamine, Desipramine, Imp-A, Iminodibenzyl and the degradation impurities (Fig 1) were well separated with a resolution greater than 4. The system suitability results are captured in table 1 and the developed UPLC method was found to be specific for Imipramine, its known impurities and degradation impurities (Fig 2).

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 Imipramine was demonstrated in the presence of its known impurities, namely Depramine, Desipramine, Imp-A, Iminodibenzyl and its degradation products. Thorough forced degradation studies were carried out on Imipramine 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 [35]. The thermal stress was done at 105°C for 10 days. The acid, base stress was performed with 5.0 N HCl and 1.0 N NaOH on Imipramine for 4 days at ambient temperature (25±2°C). Water hydrolysis was performed for 5 days at ambient temperature. The oxidation stress was done with 5 % hydrogen peroxide for 4 days at ambient temperature [36-37]. All the stressed samples were quantified for Imipramine and its impurities. Peak purity of stressed samples of Imipramine and the spiked solution of Imipramine with its known and degradation impurities were checked by waters acquity diode array detector (DAD). Additionally the unknown degradation products DG1 (acid degradation) and DG2 (oxidative degradation) formed were identified by NMR and mass 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 Imipramine were performed with 0.15 % *w/w* of each DG1, Depramine, Desipramine, DG2, Imp-A and Iminodibenzyl to the reference of TAC. Quantification of individual impurities and Imipramine 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 Imipramine with its related impurities at LOQ level and calculating the percentage RSD for the areas of Imipramine and its related impurities. Accuracy at LOQ level was also carried out by preparing three recovery solutions of Imipramine 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 Imipramine with its related 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 Imipramine 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 Imipramine.

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 DG1, Depramine, Desipramine, DG2, Imp-A and Iminodibenzyl 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. 800, 1000 and 1200 µg mL-1 of Imipramine, corresponding to 80, 100 and 120 % *w/w* of the TAC. The percentage recovery at each level was calculated against the Imipramine standard, considered 99.3 % *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 Imipramine and the resolution between Imipramine, DG1, Depramine, Desipramine, DG2, Imp-A and Iminodibenzyl 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 252 nm and 256 nm, instead of 254 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: 0/35, 10/90 and 0/45, 10/90, instead of 0/40, 10/90.

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 Imipramine was studied for 48 h at room temperature. The reference standard of Imipramine and the sample spiked with impurities at specification level were injected every 6 h. The content of impurities and Imipramine 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 6 h. During the study period, the prepared mobile phase remained unchanged. The recovery of the Imipramine 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

Slight degradation of drug substance was observed in water, base hydrolysis, thermal and photolytic stress conditions. Significant degradation of drug substance was observed in acidic hydrolysis and oxidative stress conditions. The acid & oxidative degradation samples were analyzed using the developed UPLC method, revealed the formation of two major degradation unknown impurities, one in acid degradation at RT 2.4 min (RRT 0.45) and another in oxidative degradation at RT 4.8 min (RRT0.92). These two degradation impurities were isolated using mentioned semi preparative HPLC conditions. These degradation impurities were marked as DG1 (acid degradation) and DG2 (oxidative degradation). UPLC chromatogram of Imipramine with its known impurities and degradation impurities is shown in Fig. 1

The peak purity factor was within the threshold limit for all stressed samples, which demonstrates the specificity of the Imipramine peak (Fig 3-acid degradation, and Fig 4-oxidative degradation). The assay of Imipramine was unaffected in the presence of Depramine, Desipramine, Imp-A, Iminodibenzyl and its degradation products, the mass balance of stressed samples was between 99.1 and 100.2 % *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 2.

Structural elucidation of impurities

The chemical structures of degradation impurities DG1 and DG2, the numbering scheme for NMR, MS spectra and the probable molecular formula by HR-MS and the ^{1}H, 2D NMR and ^{13}CNMR spectral data for DG1 & DG2 are shown in Table 3 and Table 4 respectively. The structural elucidation of Imipramine has been described in Table 5.

Acid degradation impurity - DG1

ESI mass spectrum of DG1 displayed a protonated molecular ion at m/z 281 [M+H] in positive ion mode, indicating the mass of this impurity as 280 which was same as that of Imipramine. The HR-MS spectrum was showing the probable molecular formula for the impurity as C_{18} H_{20} N_2 O where as for Imipramine it was C_{19} H_{24} N_2. The molecular formula of DG1 was showing the loss of 4 protons and one carbon, and addition of one oxygen when compared to Imipramine. In DG1, the signals corresponding to 7 and 7' of Imipramine (Table 5) CH2-CH2 (3.056 ppm, s, 4H) protons were disappeared in ^{1}HNMR and the corresponding carbon signals (31.510 ppm) were also disappeared in ^{13}CMR. The DG1 impurity exhibited one characteristic peak at 171.213 ppm in ^{13}CNMR indicating the presence of carbonyl group, which is further supported by a characteristic absorption band of DG1 at 1702 cm^{-1} in FT-IR spectrum and extended conjugation by UV. There were no changes in the proton or carbon shifts of N,N-dimethyl isopropyl group when compared to Imipramine. Based on the above spectral data the molecule formula of DG1 was confirmed as C_{18} H_{20} N_2 O and the corresponding structure was characterized as 10-(3-(dimethylamino)propyl)acridin-9(10H)-one**.** The detailed assignments of proton, carbon and 2D correlations of DG1 impurity are presented in table 3. The MS spectra and the probable molecular formula by HR-MS, and the ^{1}H, 2D NMR and ^{13}C NMR spectral data of DG1 are presented in Fig 5.

Oxidative degradation impurity - DG2

ESI mass spectrum of DG2 displayed a protonated molecular ion at m/z 297 [M+H] in positive ion mode, indicating the mass of this impurity as 296 which was 16 amu more than that of Imipramine. The HR-MS spectrum was showing the probable molecular formula for the impurity as C_{19} H_{24} N_2 O where as for Imipramine C_{19} H_{24} N_2. The molecular formula of DG2 was showing the addition of one oxygen when compared to Imipramie. The ^{1}H or ^{13}C NMR of DG2 spectra contains no additional signals when compared to Imipramine. But the proton and carbon signals of N,N-dimethyl isopropyl group were shielded more when compared to Imipramine, which reveals the addition of oxygen may be on N,N-dimethyl nitrogen – indicating the formation of mono N-oxide of Imipramie. Based on the above spectral data the molecule formula of DG2 was confirmed as C_{19} H_{24} N_2 O and the corresponding structure was characterized as 3-(10,11-dihydro-5H-dibenzo[b,f]azepin-5-yl)-N,N-dimethylpropan-1-amine oxide. The detailed assignments of proton, carbon and 2D correlations of DG2 impurity are presented in table 4. The MS spectra and the probable molecular formula by HR-MS, and the ^{1}H, 2D NMR and ^{13}C NMR spectral data of DG2 are presented in Fig 6.

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 Imipramine in the precision study, including intermediate precision, were well within 3.0 and 0.39, respectively. The percentage RSD values are reported in table 6 & table 7 for related impurities and Imipramine respectively.

Limit of detection and limit of quantification

The limit of detection of Imipramine, DG1, Depramine, Desipramine, DG2, Imp-A and Iminodibenzyl were less than or equal to 0.02 % *w/w* (of TAC) for 1 µL injection volume. The limit of quantification of Imipramine, DG1, Depramine, Desipramine, DG2, Imp-A and Iminodibenzyl were less than or equal to 0.05 % *w/w* (of TAC) for 1 µL injection volume. The percentage RSD of impurities at LOQ level were less than 3.0 and the recovery values at LOQ level were between 95.7 and 98.8. Since the dosage of the Imipramine was less than 75-300 mg 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 Imipramine and its related impurities, and precision at LOQ level are tabulated in table 6. The results of accuracy at LOQ level are tabulated in table 8.

Linearity

Excellent correlation was achieved for the regression line of Imipramine 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 Imipramine for all impurities at the optimized condition. The *Y*-intercept of each plot was below 1.9 % 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., 800 to 1200 µg mL^{-1}. An excellent correlation was obtained between the peak area and concentration of Imipramine 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 6.

Accuracy

The percentage recovery of each impurity falls in the range of 95.9 to 102.3 (Table 8). The individual assay value at each level in triplicate is close to the derived true value (Table 7). 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 Imipramine was less than 1.3 and the resolution for the critical pair DG2 and Imipramine was greater than 3.8, and for the critical pair Imipramine and Imp-A was greater than 3.8. 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 9.

Solution stability and mobile phase stability

The percentage RSD of the assay of Imipramine 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 99.3 and 100.4. The percentage recovery of the content of each impurity against the initial value was between 97.4 and 101.2. 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.

Conclusion

Based on the studies the degradation impurities are designated as 10-(3-(dimethylamino)propyl)acridin-9(10H)-one in acid hydrolysis and 3-(10,11-dihydro-5H-dibenzo[b,f]azepin-5-yl)-N,N-dimethylpropan-1-amineoxide in oxidative degradation. The developed simple UPLC method for related substance and assay determination of Imipramine 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 Imipramine 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.

Acknowledgement

The authors wish to thank the management of Dr. Reddy's Group for supporting this work. Authors wish to acknowledge the process research group for providing the samples for our research and also wish to thank colleagues in separation science division of analytical research of Custom Pharmaceutical Services for their co-operation in carrying out this work.

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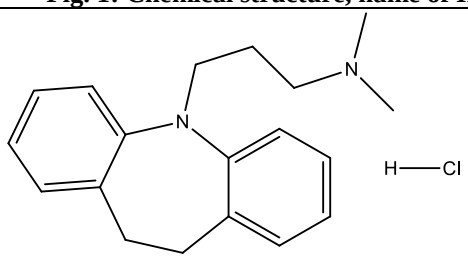
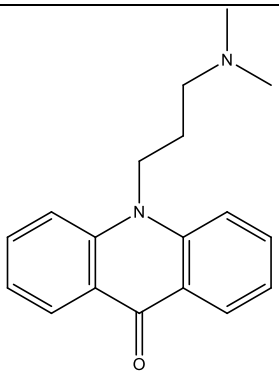
Fig. 1: Chemical structure, name of Imipramine and its impurities

Imipramine		3-(10,11-dihydro-5H-dibenzo[b,f]azepin-5-yl)-N,N-dimethylpropan-1-amine monohydrochloride
DG1		10-(3-(dimethylamino)propyl)acridin-9(10H)-one

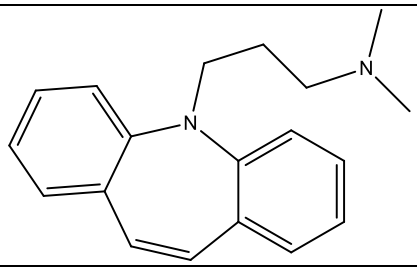
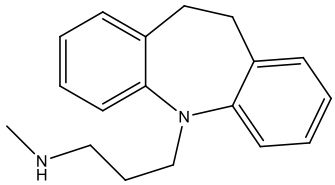
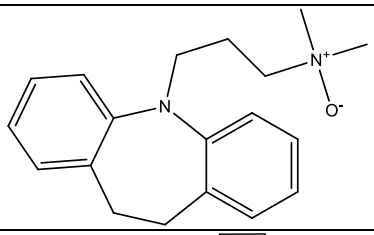
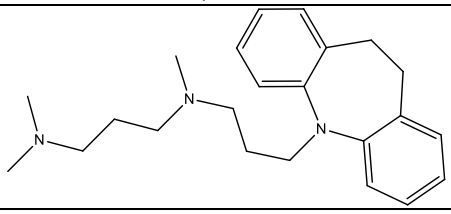
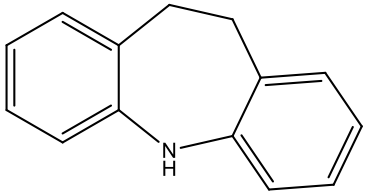
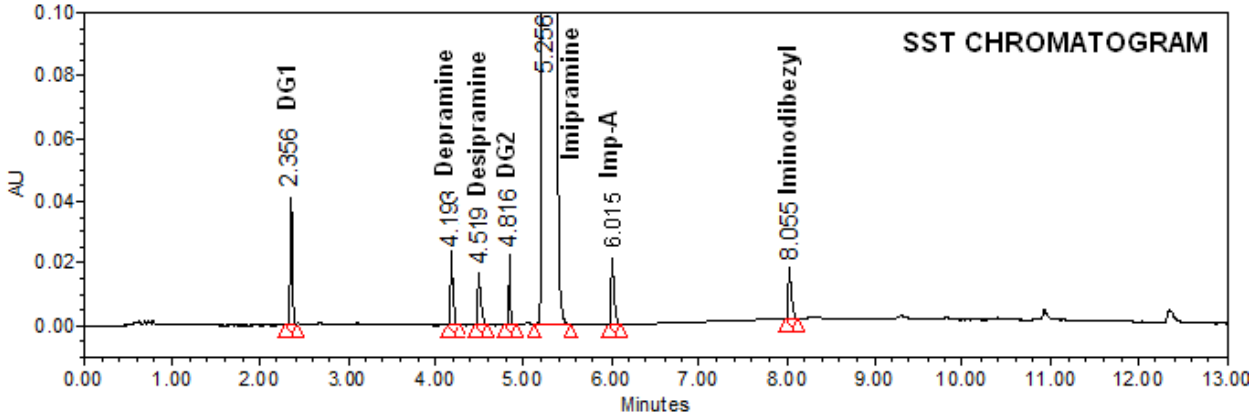
Depramine		3-(5H-Dibenzo[b,f]azepin-5-yl)-N,N-dimethylpropan-1-amine
Desipramie		3-(10,11-dihydro-5H-dibenzo[b,f]azepin-5-yl)-N-methylpropan-1-amine
DG2		3-(10,11-dihydro-5H-dibenzo[b,f]azepin-5-yl)-N,N-dimethylpropan-1-amine oxide
Imp-A		N-[3-(10,11-Dihydro-5H-dibenzo-[b,f]azepin-5-yl)propyl]-N,N',N'-trimethylpropane-1,3-diamine
Iminodibenzyl		10,11-Dihydro-5H-dibenz[b,f]azepine

Fig. 2: Typical chromatograms of system suitability in developed UPLC method



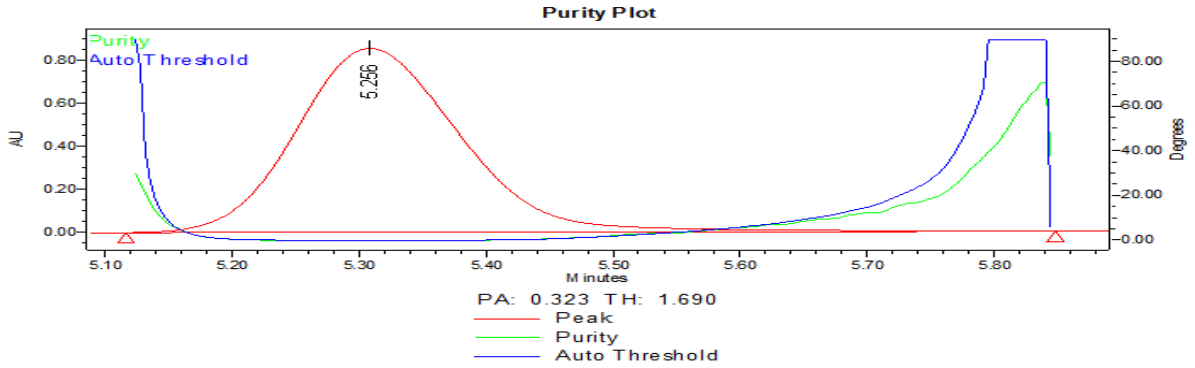


Fig. 3: Typical chromatogram acid degradation

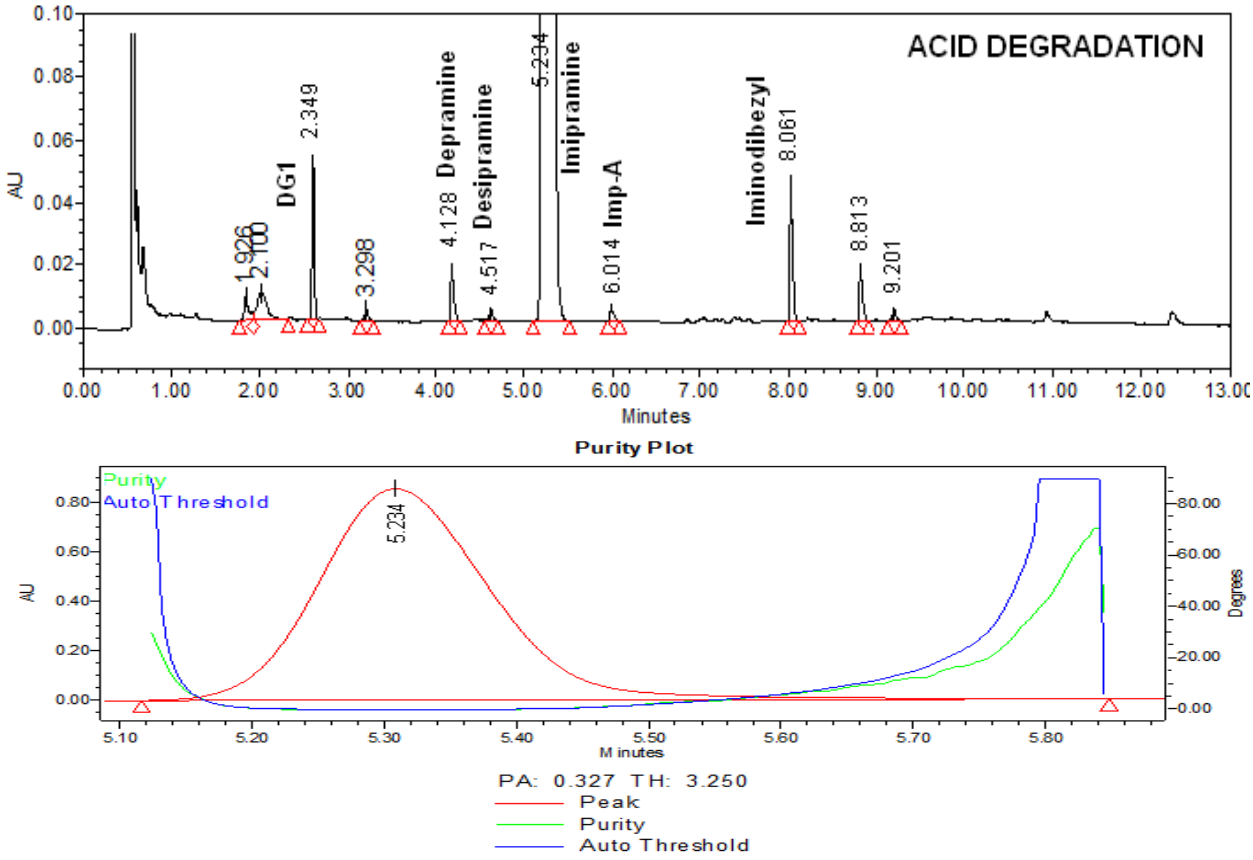


Fig. 4: Typical chromatogram oxidative degradation

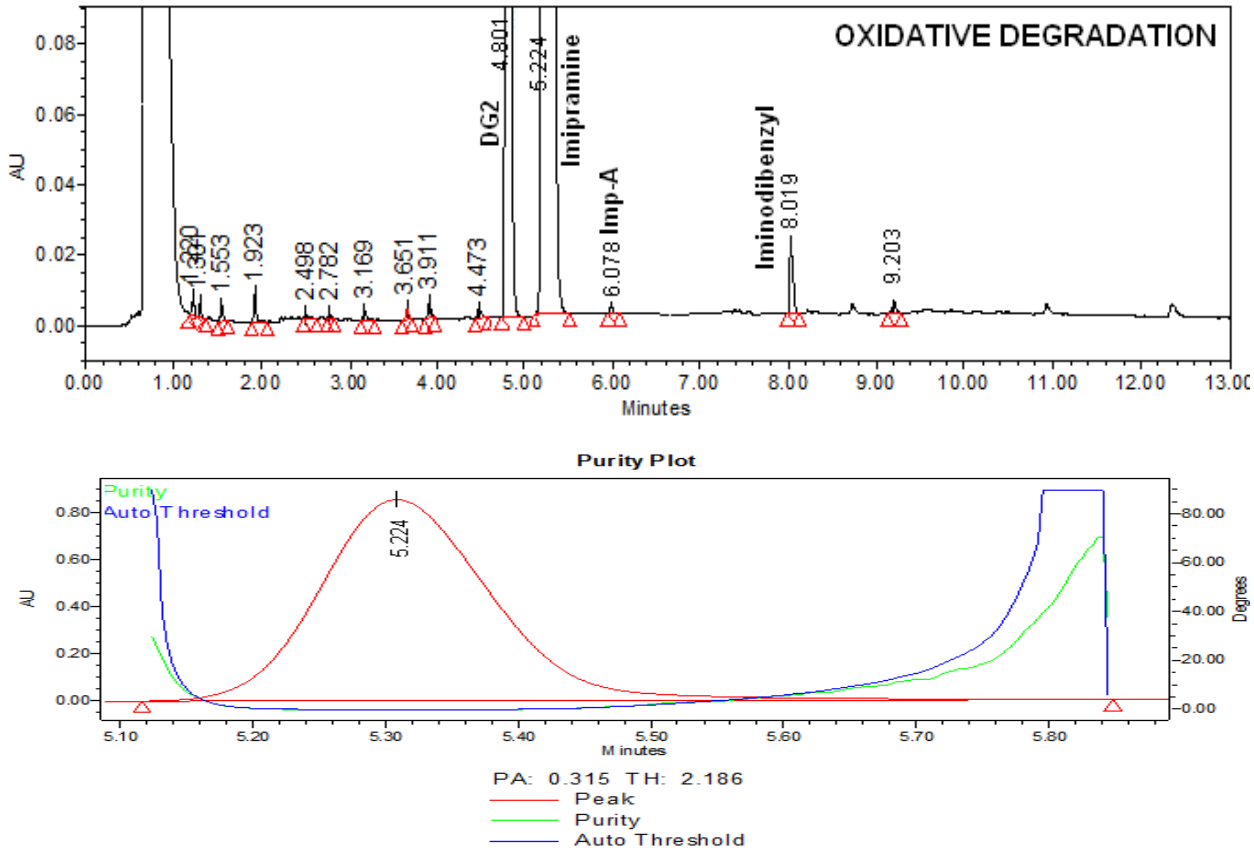
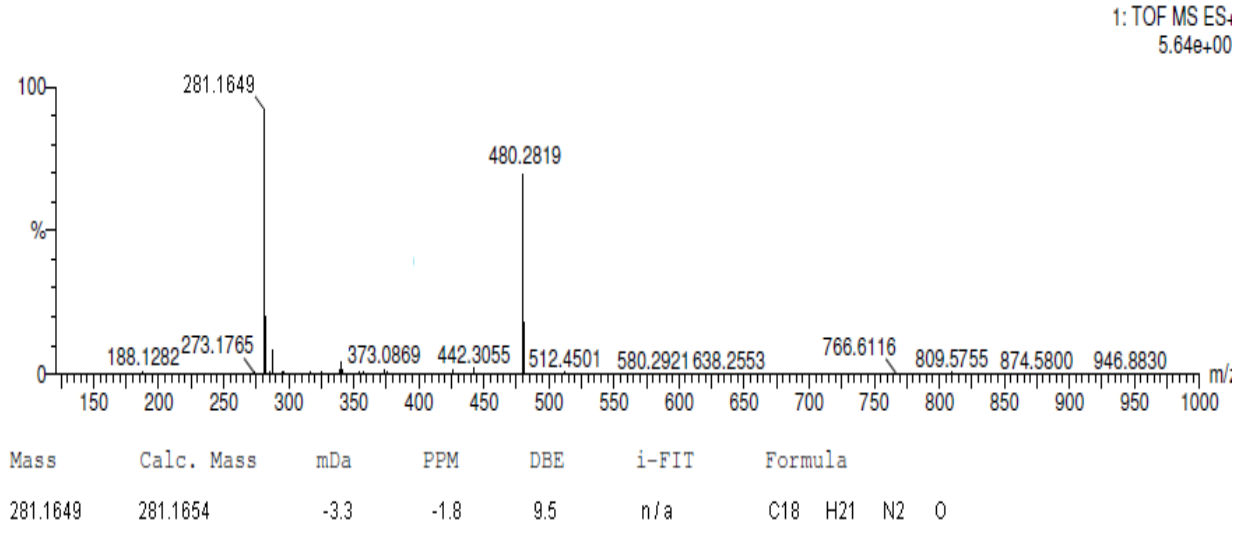


Fig. 5: Typical HR-MS, ^{1}HNMR, ^{13}CNMR, COESY and HSQC data of DG1



Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
281.1649	281.1654	-3.3	-1.8	9.5	n/a	C18 H21 N2 O

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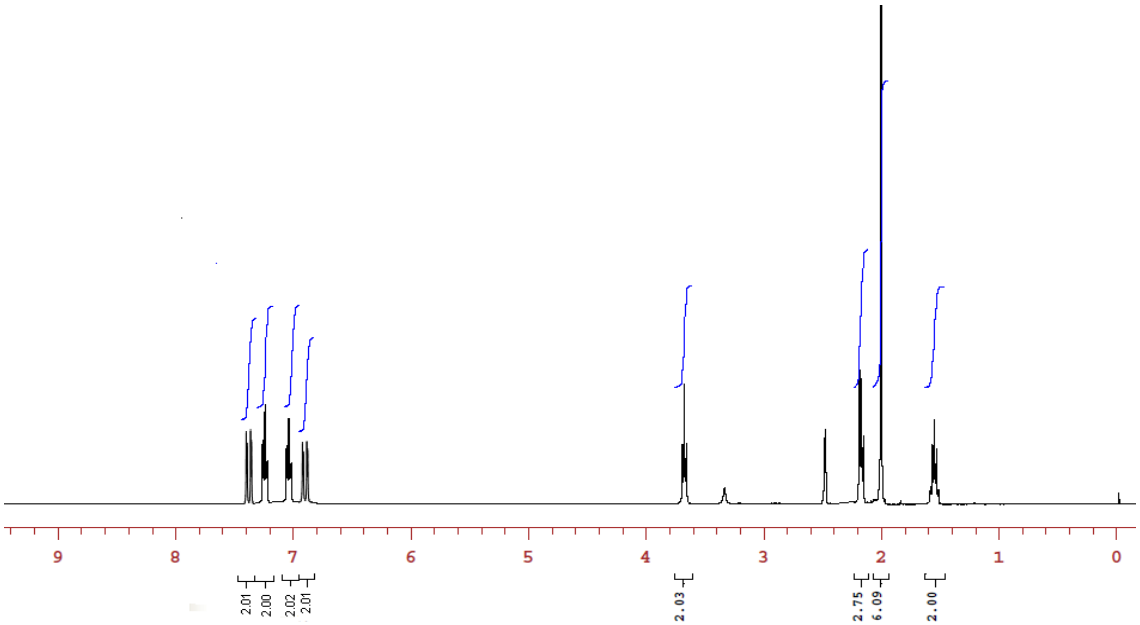
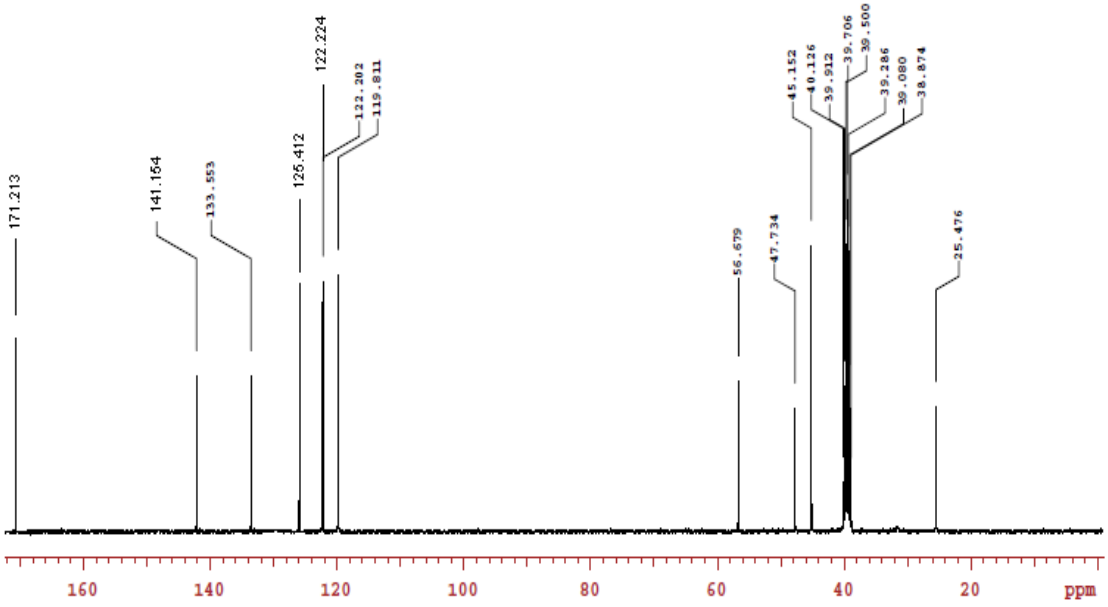
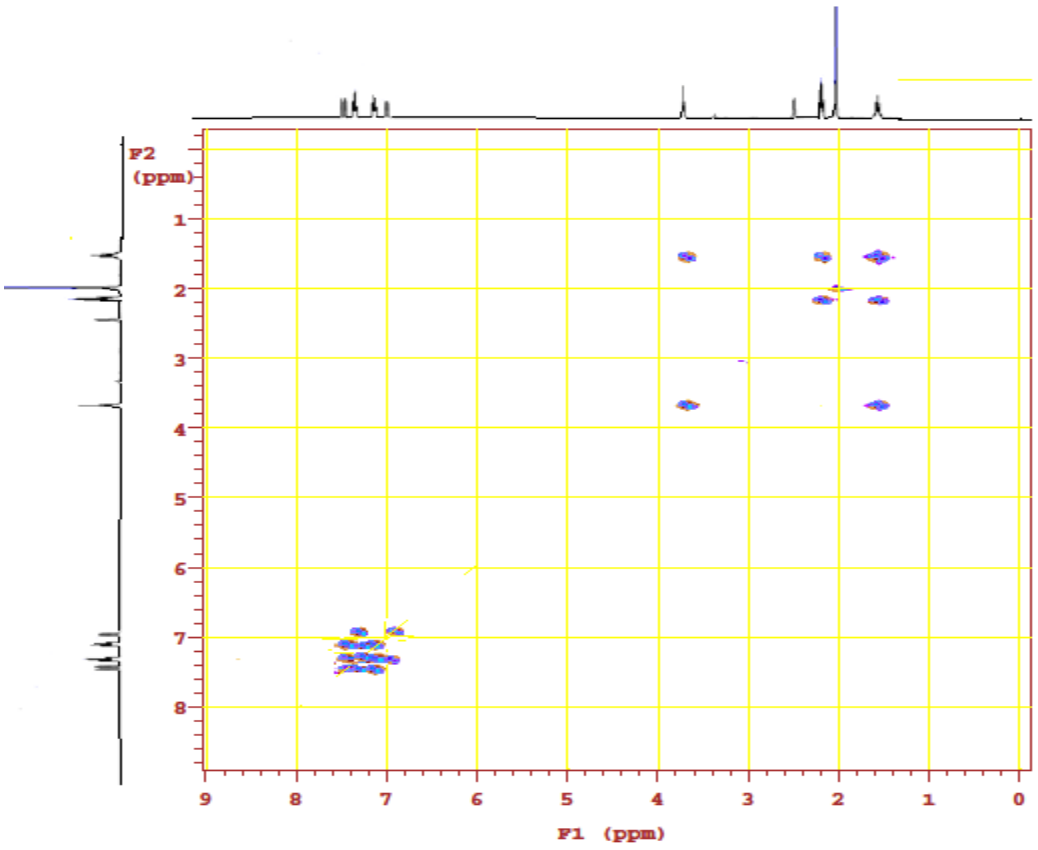




[Vukkum *et al.*, 5(11): Nov., 2014:3986-4006]
ISSN: 0976-7126



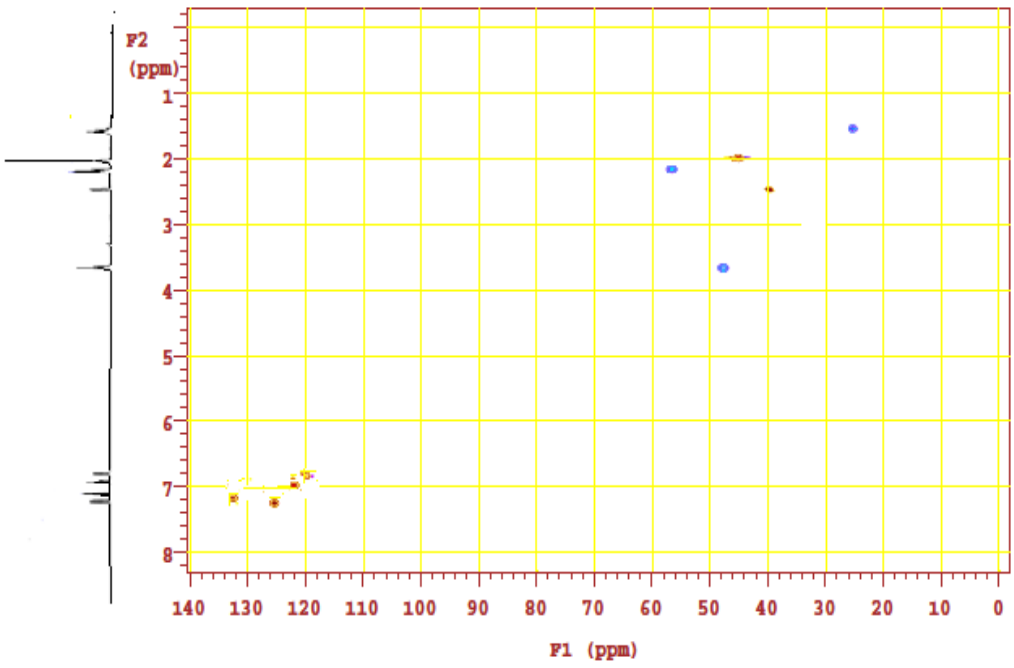
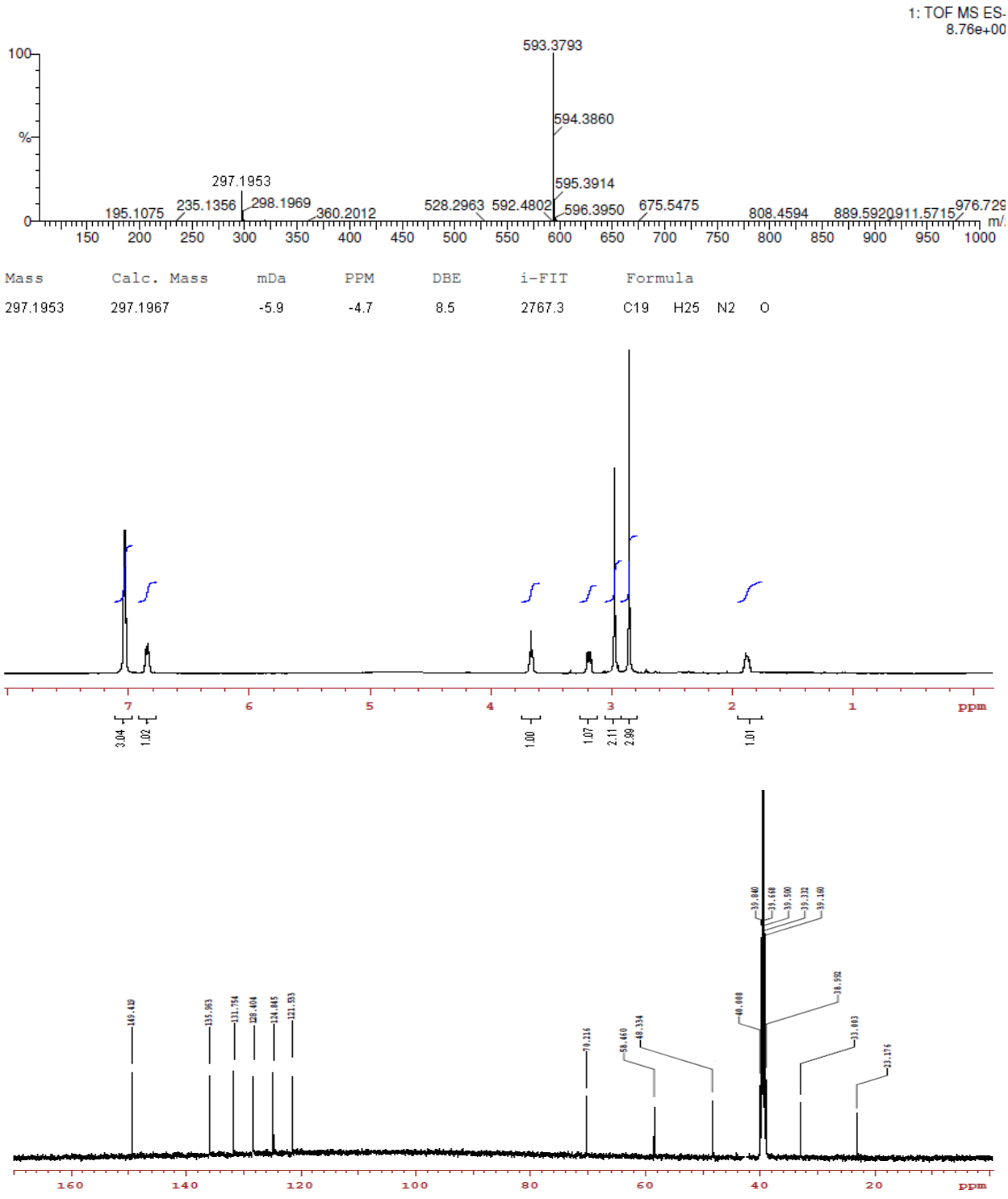
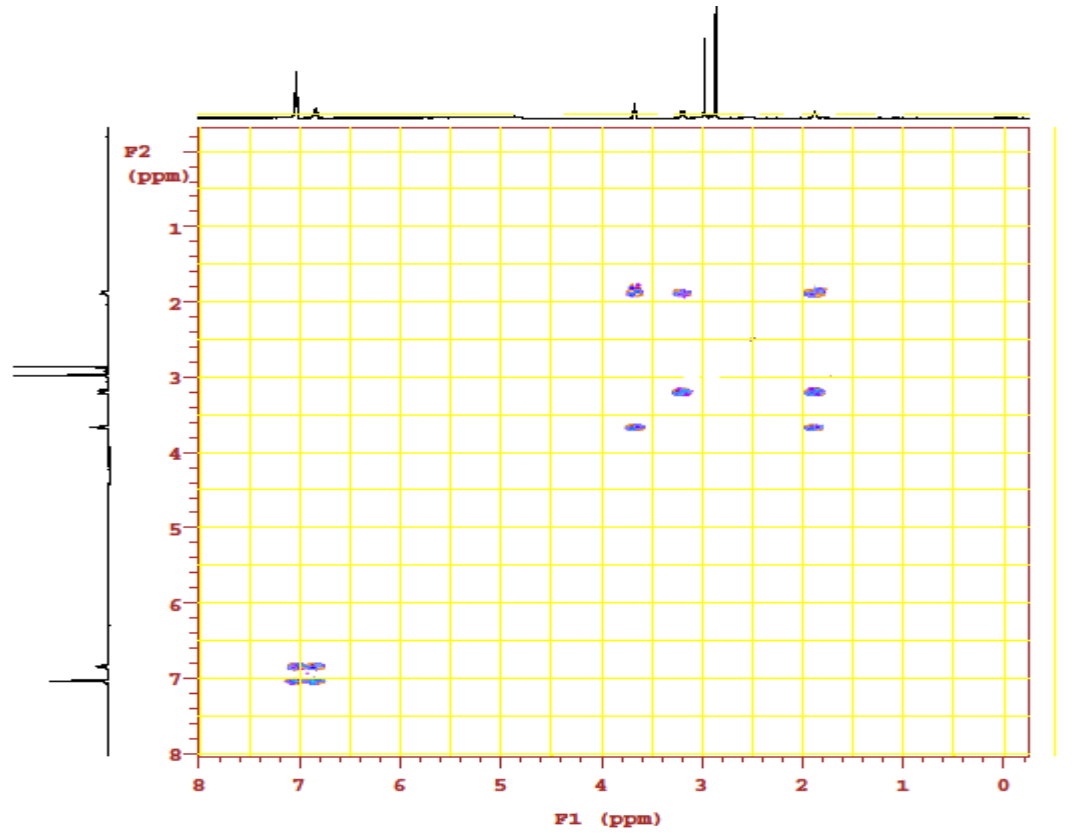


Fig. 6: Typical HR-MS, ^{1}HNMR, ^{13}CNMR, COESY and HSQC data of DG2




F2
(ppm)
1
2
3
4
5
6
7
8
8
7
6
5
4
3
2
1
0
F1 (ppm)

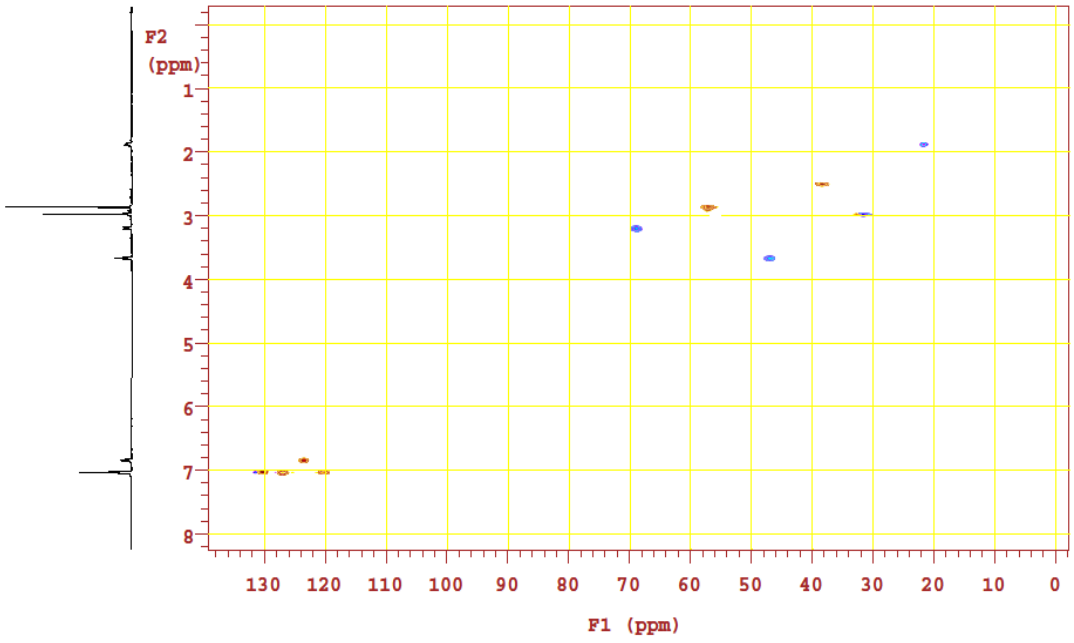

F2
(ppm)
1
2
3
4
5
6
7
8
130
120
110
100
90
80
70
60
50
40
30
20
10
0
F1 (ppm)

Table 1: Results of system suitability test

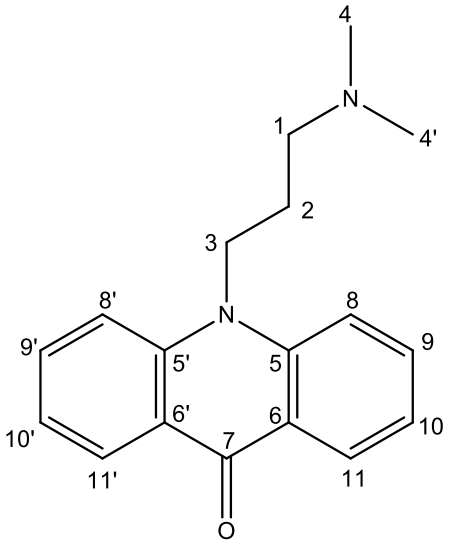
Compound *(n = 3)*	Retention time (*RT*)	Relative retention time (*RRT*)	Capacity factor (*k'*)	Resolution *(R_s)*	USP Tailing factor *(T)*	No. of Theoretical plates *(N-* Tangent method)
DG1	2.4	0.45±0.01	10.8±0.03	--	1.0±0.01	21239
Depramine	4.2	0.80±0.01	20.0±0.07	27±0.02	1.1±0.01	61025
Desipramine	4.5	0.86±0.01	21.6±0.13	5.2±0.14	1.2±0.04	59005
DG2	4.8	0.92±0.00	23.1±0.01	4.4±0.09	1.1±0.02	70476
Imipramine	5.3	1.00±0.00	25.3±0.12	4.5±0.06	1.2±0.04	34214
Imp-A	6.0	1.14±0.01	29.3±0.11	6.5±0.02	1.3±0.03	111268
Iminodibenzyl	8.1	1.53±0.00	39.3±0.04	25±0.08	1.1±0.01	171774

Table 2: **Summary of forced degradation results**

Stress condition	Duration	Purity of Analyte after degradation	Assay of Analyte after degradation	Observations
Unstressed sample	--	99.8	99.9	--
Water hydrolysis	5 days	99.1	99.0	Slight degradation was observed. Known impurity Iminodibenzyl was formed
Acid hydrolysis (5N HCl)	4 days	89.1	89.6	Significant degradation was observed. Unknown impurity DG1 and Iminodibezyl were formed as major degradation products
Base hydrolysis (1N NaOH)	4 days	99.2	99.0	Slight degradation was observed. Known impurity Iminodibenzyl and Imp-A were formed

Oxidation (5% H2 O2)	4 days	85.3	85.9	Significant degradation was observed. Unknown impurity DG2 and Iminodibezyl were formed as major degradation products
Thermal (105° C)	10 days	98.9	99.1	Slight degradation was observed. Known impurity Iminodibenzyl was formed
Photolytic degradation as per ICH guidelines both in UV & Visible	11 days	98.4	98.3	Slight degradation was observed. Known impurity Iminodibenzyl was formed

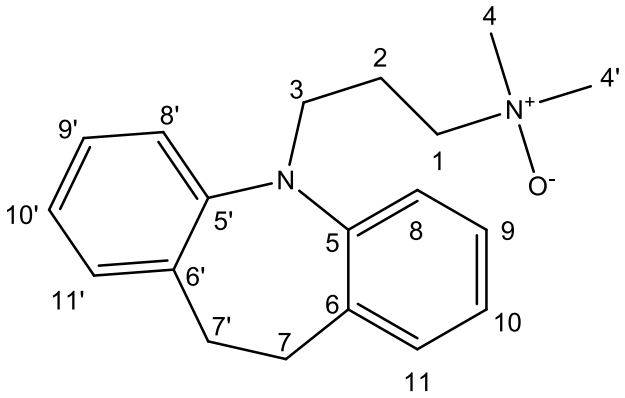
Table 3: Structural characterization of acid degradation impurity DG1



Position[a]	^{1}H, multiplicity	δ (ppm)	^{13}C	gHSQC (position, δ)	gDQ-COSY (position, δ)	Assignment
1	2H, t	2.173	56.679	(1H, 2.173)	(2H, 1.549)	CH2
2	2H, m	1.549	25.476	(2H, 1.549)	(1H, 2.173) (3H, 3.675)	CH2

3	2H, t	3.675	47.734	(3H, 3.675)	(2H, 1.549)	CH2
4 & 4’	3H, s	2.002	45.152	(4H, 2.002)	--	CH3
5 & 5’	--	--	141.154	--	--	Quaternary Carbon
6 & 6’	--	--	122.224	--	--	Quaternary Carbon
7	--	--	171.213	--	--	-C=O
8 & 8’	1H, d	6.823	119.811	(8 & 8’H, 6.823)	(9 & 9’H, 7.267)	CH
9 & 9’	1H, t	7.267	133.553	(9 & 9’H, 7.267)	(8 & 8’H, 6.823) (10 & 10’H, 7.012)	CH
10 & 10’	1H, t	7.012	122.202	(10 & 10’H, 7.012)	(9 & 9’H, 7.267) (11 & 11’H, 7.401)	CH
11 & 11’	1H, d	7.401	125.412	(11 & 11’H, 7.401)	(10 & 10’H, 7.012)	CH

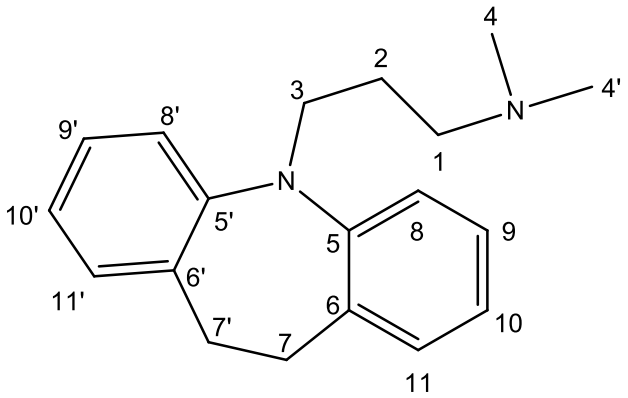
Table 4: Structural characterization of oxidative degradation impurity DG2



Position[a]	^{1}H, multiplicity	δ (ppm)	^{13}C	gHSQC (position, δ)	gDQ-COSY (position, δ)	Assignment
1	2H, t	3.189	70.216	(1H, 3.189)	(2H, 1.886)	CH2
2	2H, m	1.886	23.176	(2H, 1.886)	(1H, 3.189) (3H, 3.666)	CH2
3	2H, t	3.666	48.334	(3H, 3.666)	(2H, 1.886)	CH2
4 & 4’	3H, s	2.853	58.460	(4H, 2.853)	--	CH3

5 & 5’	--	--	149.419	--	--	Quaternary Carbon
6 & 6’	--	--	135.963	--	--	Quaternary Carbon
7 & 7’	2H, s	2.974	33.003	(7 & 7’H, 2.974)		CH2
8 & 8’	1H, m	6.845	124.845	(8 & 8’H, 6.845)	(9 & 9’H, 7.038)	CH
9 & 9’	1H, m	7.038	128.404	(9 & 9’H, 7.038)	(8 & 8’H, 6.845) (10 & 10’H, 7.038)	CH
10 & 10’	1H, m	7.038	121.533	(10 & 10’H, 7.038)	(9 & 9’H, 7.038) (11 & 11’H, 7.038)	CH
11 & 11’	1H, m	7.038	131.754	(11 & 11’H, 7.038)	(10 & 10’H, 7.038)	CH

Table 5: Structural characterization of Imipramine



Position[a]	^{1}H, multiplicity	δ (ppm)	^{13}C	gHSQC (position, δ)	gDQ-COSY (position, δ)	Assignment
1	2H, t	2.186	56.678	(1H, 2.186	(2H, 1.549)	CH2
2	2H, m	1.549	25.475	(2H, 1.549)	(1H, 2.186) (3H, 3.675)	CH2
3	2H, t	3.675	47.731	(3H, 3.675)	(2H, 1.549)	CH2
4 & 4’	3H, s	2.001	45.151	(4H, 2.001)	--	CH3
5 & 5’	--	--	148.135	--	--	Quaternary Carbon
6 & 6’	--	--	133.553	--	--	Quaternary Carbon

7 & 7'	2H, s	3.056	31.510	(7 & 7'H, 3.056)	--	CH2
8 & 8'	1H, m	6.861	124.202	(8 & 8'H, 6.861)	(9 & 9'H, 7.097)	CH
9 & 9'	1H, m	7.097	128.655	(9 & 9'H, 7.097)	(8 & 8'H, 6.861) (10 & 10'H, 7.097)	CH
10 & 10'	1H, m	7.097	121.813	(10 & 10'H, 7.097)	(9 & 9'H, 7.097) (11 & 11'H, 7.097)	CH
11 & 11'	1H, m	7.097	131.329	(11 & 11'H, 7.097)	(10 & 10'H, 7.097)	CH

Table 6: Results of validation parameters for related impurities

Parameter	Imipramine	DG1	Depramine	Desipramine	DG2	Imp-A	Iminodiben-zyl
LOD (% *w/w* with respect to TAC)	0.01	0.01	0.02	0.02	0.01	0.02	0.01
LOD (ng mL^{-1})	130	132	180	175	149	180	145
LOQ (% *w/w* with respect to TAC)	0.04	0.04	0.05	0.05	0.04	0.05	0.04
LOQ (ng mL^{-1})	412.3	426.4	501.1	498.7	448.8	526.7	415.9
Linearity							
Slope (*m*)	401235	485494	461420	405247	453396	421297	409260
Intercept (*C*)	652	323	526	456	752	158	961
% *Y*-intercept	1.24	0.98	1.65	1.89	1.32	0.97	1.16
Correlation coefficient	0.9998	0.9997	0.9995	0.9992	0.9997	0.9994	0.9994
Precision at LOQ level (%RSD for $n = 6$)	2.23	0.64	1.39	2.92	0.98	1.53	0.97
Precision (%RSD for $n = 6$)	--	1.12	1.06	2.98	1.42	2.31	1.78
Ruggedness(%RSD for $n = 6$)	--	1.97	1.05	2.45	1.07	2.49	1.33
Relative response factor	1.00	1.21	1.15	1.01	1.13	1.05	1.02

Table 7: Results of validation for imipramine at assay level

Parameter	Imipramine
Linearity	
Slope (*m*)	41569823
Intercept (*C*)	68156
% *Y*-intercept	0.22
Correlation coefficient	0.9998
Precision (%RSD for $n = 6$)	0.14
Ruggedness(%RSD for $n = 6$)	0.39
% Recovery for $n = 3$	
80% level	99.3 ± 0.12
100% level	99.7 ± 0.32
120% level	99.1 ± 0.15

Table 8: **Accuracy for related substances**

Amount spiked	% Recovery for *n = 3*					
	DG1	Depramine	Desipramine	DG2	Imp-A	Iminodibenzyl
LOQ	96.4± 0.23	97.1± 0.12	98.3± 0.31	96.1± 0.45	98.3± 0.47	94.9± 0.11
0.075 % *w/w* of TAC	97.4± 0.51	98.2± 0.81	98.7± 0.43	96.4± 0.57	98.1± 0.58	96.6± 0.69
0.15 % *w/w* of TAC	97.8± 0.23	98.7± 0.79	98.4± 0.19	99.7± 0.32	99.3± 0.57	99.8± 0.71
0.225 % *w/w* of TAC	102.1± 0.21	101.5± 0.36	99.2± 0.78	99.3± 0.91	99.5± 0.45	99.8± 0.68

Table 9: **Results of robustness parameters**

Parameter	Actual value	Changed value	No. of Theoretical plates *(N-* Tangent method)	USP Tailing factor (*T*)	Resolution *(R_s)* between DG2 and Imipramine	Resolution *(R_s)* between Imipramine and Imp-A
Flow rate	0.3 mL min^{-1}	0.27 mL min^{-1}	34001	1.3	4.1	4.5
		0.33 mL min^{-1}	41123	1.2	3.9	3.8
Wave length	254 nm	252 nm	33457	1.2	4.3	4.7
		256 nm	34125	1.2	4.3	4.7
Temperature	40°C	35°C	31278	1.2	3.9	4.1
		45°C	44951	1.1	4.8	4.8
Time/% mobile phase B	0/40, 10/90	0/35, 10/90	32568	1.2	5.1	5.1
		0/45, 10/90	39658	1.2	3.8	4.3

How to cite this article
Vukkum P., Babu J.M. and Muralikrishna R. (2014). Isolation and structural characterization of Imipramine hydrochloride degradation impurities and development of stability-indicating UPLC method. *Int. J. Pharm. Life Sci.*, 5(11):3986-4006.
Source of Support: Nil; Conflict of Interest: None declared
Received: 09.10.14; Revised: 15.10.14; Accepted:28.10.14