3.1. SELECTION OF DRUG

First approved in early 1960's, Cyproheptadine hydrochloride (CPH) has been one of the widely used antihistaminic drug without any serious side effects reported till date. It is also widely used as appetite stimulant as it competes with serotonin at its receptor sites in intestine. Its NDA was filed by Merck in 1961. Generic version of the drug is also widely available. Due to its induction in early era, most of references available for various studies are approximately 30 years old. Despite the fact that sufficient literature is available for the said drug substance, till date no HPLC-PDA based stability indicating analytical method along with LC/MS/MS, NMR characterization for major degradation product have been reported for the drug substance CPH. The present research work describes development of a stability indicating analytical method for CPH. CPH showed sufficient degradation and UV visible degradation products under acid and base stress conditions. The major degradation product formed in acid degradation condition was isolated, characterized and identified.

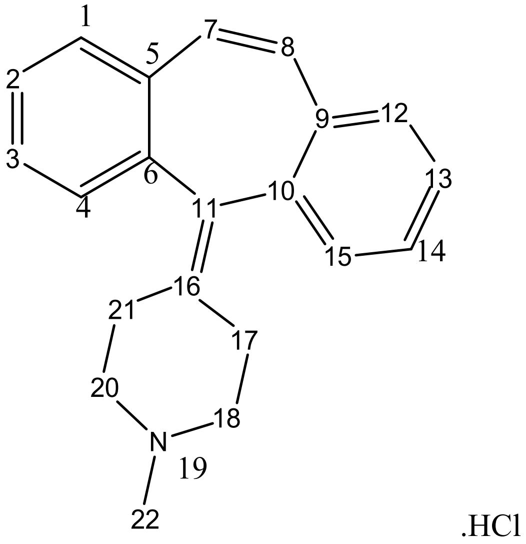
3.2 DRUG PROFILE [2]

IUPAC Name: 4-(5Hdibenzo [a, d] - cyclohepten-5-ylidene)-1-methylpiperidine hydrochloride.

Molecular Formula: $C_{21}H_{21}N.HCl$

Molecular Weight: 323.86 g/mol

Chemical Structure:



3.1: Structure of CPH

Appearance: White to slightly yellowish crystalline solid

Melting point: 112-113°C

pKa: 8.05

Log p: 4.38

Solubility: Freely soluble in water, freely soluble in methanol, sparingly soluble in ethanol, soluble in chloroform, and practically insoluble in ether

Drug Category: Anti-histaminic

Mechanism of action: Histamine H1 blocker. CPH does not prevent the release of histamine but rather competes with free histamine for binding at HA-receptor sites. [3]

Uses: Anti pruritic, appetite stimulant, anti allergic, Cushing syndrome, Nelson's syndrome, Parkinsonism etc. [3]

Marketed Formulation:
Periactin tablets having 4 mg content of CPH (Merck Sharp and Dohmes)
Heptidin tablets having 4 mg content of CPH (Unison Pharmaceuticals Pvt. Ltd)
Ciplactin tablets having 4 mg content of CPH (Cipla)
Cypron tablets having 4 mg content of CPH (Kable)

3.3 LITERATURE REVIEW

The analytical method in Indian Pharmacopoeia [4] describes an UVspectrophotometric method for its assay in tablet. The United States Pharmacoepoeia [5] describes titrimetric method for assay of CPH. Several other analytical methods have also been reported for the analysis of CPH in its bulk and dosage forms. Application of Ion pair complexometric UV-spectrophotometry for the assay of CPH using bromophenol blue has been reported by Kanakapura Basavaiah. [6] A colorimetric method for the assay of CPH in tablets was reported by Sayanna etal. [7] Extractive colorimetric method was reported by Sane et al. [8] A liquid chromatographic-tandem mass spectrometric (LC-MS/MS) method was reported by Feas et al for the assay of CPH in syrup formulations. [9] Gaschromatographic procedures were also developed for the assay of CPH in tablets. [10, 11] Novak et al, have reported a HPLC method for quantification of CPH in serum or plasma. [12] Another quantification procedure for CPH in plasma and urine by HPLC has been reported by Foda et al. [13]. Another HPLC method for determination of CPH was reported by Burrows etal. [14] RP-HPLC method for the determination of CPH in urine was developed by Kountourellis and Ebete. [15] Another GLC method for determination of CPH in urine and plasma using nitrogen sensitive detector has been reported by Hucker and Hutt. [16] HPLC method in combination with chemometrics was also reported for analysis of CPH in multidrug formulation. [23] A bioanalytical HPLC method for CPH using DLLME procedure for extraction of CPH from human urine was also reported. [24] At the time of undertaking the study no stability indicating analytical studies were available for the said drug but very recently one stability indicating analytical method using fluorescence detector was reported along with degradation kinetic studies for oxidative stress condition. [25] Also a comprehensive investigation on chromatographic methods for CPH was done in recent past mainly focusing on HPLC and TLC methods. [26] Along with it a stability indicating first derivative spectroflorimetric method for determination of CPH along with its Oxidative degradation product was also reported. [27] A comprehensive spectrophotometric study was also reported for CPH. [29]

3.4. SECTION –A

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPLC METHOD FOR CYPROHEPTADINE HCl

3.4.1 Experimental

3.4.1.1 Chemicals and materials

Reference standard of CPH was obtained as a gift sample from HealthCare Pharmaceuticals Pvt. Ltd. (Vadodara, India). The marketed formulation of CPH tablets with brand name ‘Heptidin’ tablets manufactured by Unison Pharmaceuticals Pvt. Ltd were procured from local pharmacy. HPLC grade acetonitrile and methanol were procured from Fischer Scientific Pvt Ltd. (India). Ammonium formate and formic acid were purchased from Lobachem chemicals Pvt. Ltd (Mumbai, India) and Merck (India) respectively. Analytical grade toluene, dichloromethane, diethylether, ethylacetate, acetone, pyridine, methanol, water and acetic acid were procured from Spectrochem Pvt. Ltd., Mumbai. Silica gel-G was also procured from Spectrochem Pvt. Ltd., Mumbai. Unless otherwise specified, all solutions were filtered through a 0.2 µm Nylon 6, 6 membrane filter, Ultipor® N66® from Pall Life Sciences, USA; prior to use. Analytical grade hydrochloric acid (HCl) and sodium hydroxide (NaOH) were procured from SD Fine chem. Ltd., Mumbai, India. Hydrogen peroxide (H_2O_2) was procured from Fischer Ltd., India.

3.4.1.2 Equipments and analysis conditions (UV, HPLC-PDA)

Precision water/oil baths equipped with temperature controller were used for degradation studies for acidic, basic hydrolysis and oxidative degradation conditions. Photolytic degradation study was carried out in a photo-stability chamber (Thermolab Scientific Equipments Pvt Ltd, Vadodara) equipped with a light bank consisting of four UV (OSRAM L73) and fluorescent (OSRAM L20) lamps, that complied with specifications prescribed in the ICH guideline Q1B. The system is capable of controlling specific temperature and humidity (±2^0C and ± 5% RH). Other equipments used were an ultrasonic bath (Analab Scintific Instruments Pvt Ltd, Vadodara), precision analytical balance (A X 120, by Shimadzu Corporation analytical and measuring Instruments division, Kyoto, Japan), pH Meter (Labindia Instruments Pvt Ltd, Navi Mumbai). Related calculations were done by Microsoft Excel 2010.

Suitable wavelength for chromatographic separation and estimation of drug was identified by scanning over the range of 200–400 nm with a Shimadzu UV-1700 double beam spectrophotometer (Shimadzu, Japan).

Chromatographic analysis was carried out on a Waters, Ahmedabad (from Waters Acquity Corporation, Milford, MA, USA) and consisting of a gradient pump, PDA detector, a manual injection facility with 20 µl fixed loop, low pressure gradient flow control valve , column oven, solvent delivery module. The chromatographic analysis was performed using Empower 3 software on a Hypersil BDS C_{18} column (250×4.6 mm, 5 µm particle size). The analysis was performed at ambient temperature with injection volume of 20 µl. The mobile phase was filtered through 0.2 µm disposable filters from Ultipore®, PALL life sciences (40 mm) and degassed by ultrasonic vibrations prior to use.

3.4.1.3 Preparation of calibration solutions and quality control samples

The standard stock solution of CPH (1000 µg/ml) was prepared by dissolving 10 mg of its hydrochloride salt in 10 ml of acetonitrile: DDW (10:90, v/v) solution in a 10 ml volumetric flask. Then working stock solution of CPH (100.0 µg/ml) was prepared in acetonitrile: DDW (10:90, v/v) respectively by dissolving standard stock solution 10 times by taking aliquot of 2.5 ml of standard stock solution in 25 ml volumetric flask and dissolving it up to mark to get 100 µg/ml of working stock solution. The calibration solutions (CS's) for CPH were prepared in range of 3-18 µg/ml by taking 0.3, 0.6, 0.9, 1.2, 1.5 and 1.8 ml aliquots of working standard solution in 10 ml volumetric flask and making up to mark with mobile phase finalized for the study to get 3 – 18 µg/ml of calibration solutions.

3.4.1.4 Preparation of buffer solution

Ammonium formate (20 mM) was prepared by dissolving 0.63 gm of anhydrous ammonium acetate in 500 ml of double distilled water and adjusted to pH 5.5 using formic acid which was finally filtered with 0.2 μm Nylon membrane filter and degassed by ultra-sonication for 5 minutes.

3.4.1.5 Preparation of Degradation Products (DP's) [18, 19]

Stress degradation studies were carried out as per ICH guidelines. A preliminary degradation study was performed to gather some basic information about the stability of the API and to determine number of DPs formed under different stress conditions. Four samples were generated for every stress condition for API

i) Blank solution stored at normal conditions
ii) Blank solution subjected to stress condition in the same manner as the drug
iii) Zero time sample containing the API which was stored at normal conditions and
iv) Drug solution subjected to stress treatment.

Similar degradation conditions were applied on the marketed formulation. Then in the final study, CPH was stressed to maximum condition where 05-80% decrease in peak area of API occurred to get maximum degradation product for its isolation. A sample containing all the degradation products was used to get the final chromatogram of the optimized stability indicating analytical method.

The specific stress conditions applied were as follows:

Hydrolytic degradation studies were performed in acidic, basic and neutral conditions by heating or refluxing API in HCl, NaOH and H_2O.

As per the process flow for stability studies discussed in section 1.2, we started the hydrolytic stress degradation studies at room temperature using 0.1N of HCl as a stressor for acid hydrolytic studies and 0.1N NaOH as a stressor for basic hydrolysis studies. But no degradation of drug was observed thereby we increased the stressor conditions to 1 N of both stressor conditions. Negligible degradation was achieved on elevation of stressor concentration at room temperature. Thus, along with increased stressor, we applied elevated temperature as a combination to hydrolytic stressor conditions. Gradually the results were observed for elevated temperature using 60, 80 and 100° C. Degradation of drug was observed but it was not sufficient for development of robust stability indicating analytical method, thereby the stressor concentration was increased to 3 N for both acid (HCl) and base (NaOH) hydrolysis using 100° C as elevated temperature for the study.

For acid hydrolytic degradation, about 1 g of CPH was dissolved with 100 ml of water (10000 μg/ml), 1 ml of this stock solution was taken in a round bottom flask (RBF) and 9 ml of 3 N HCl solution was added to it (1000 μg/ml).The flask was refluxed in the high precision water bath maintained at 100°C for 5 h. After that 1 ml aliquot was withdrawn and neutralized with 3N NaOH and stored in refrigerator before analysis. For base hydrolytic degradation 1 ml of above stock solution was taken in a round bottom flask (RBF) and 9 ml of 3 N NaOH was added to it. The flask was refluxed in the high precision water bath maintained at 100°C for 3 h. After that 1 ml aliquot was withdrawn and neutralized with 3N HCl and stored in refrigerator before analysis.

Similarly for neutral hydrolytic degradation, no sufficient degradation of drug was observed at room temperature, thus it was decided to carry the analysis at elevated temperature (100°C). For the study, 0.1 ml of above stock solution was taken in a round bottom flask (RBF) and 9 ml of double distilled water was added to it. The flask was refluxed in the high precision water bath maintained at 100°C for 5 h and stored in refrigerator before analysis.

For oxidative degradation also at room temperature and at low stressor concentration (viz.,using 3% H_2O_2) analysis was carried out, but no sufficient degradation was observed thereby it was decided to carry out stress degradation studies for oxidation degradation at elevated temperature using higher stressor concentrations. For its study, 0.1 ml of above stock solution was taken in a round bottom flask (RBF) and 9 ml of 6% H_2O_2 solution added to it. The flask was refluxed in the high precision water bath maintained at 100°C for up to 12 h and stored in refrigerator before analysis.

Thermal degradation was carried out on solid CPH 100 mg spreaded on glass petridish as a thin layer about 1mm thickness in high precision oven at 60°C for up to 10 days.

For photolytic degradation also the solid drug 100 mg spread as a thin layer about 1 mm thickness in a petridish, were exposed to the UV light in the photo stability chamber. The samples were placed for 15 days during which the total UV light of about 144 Wh/m^2 and 5382 Lux.

All the degradation samples were suitably diluted with mobile phase to make final concentration of 100 μg/ml with respect to CPH and filtered through 0.2 μm nylon membrane syringe filter prior to injection in HPLC.

3.4.1.6 HPLC method and sample preparation

CPH and its degradation product were optimally resolved on a Hypersil BDS C_{18} (250 mm 4.6 mm, 5 mm) column at ambient temperature with acetonitrile-methanol-20 mM ammonium formate (pH 5.5 adjusted with 0.2% formic acid) (40:10:50, v/v/v) as the mobile phase. The mobile phase flow rate and typical pressure of the system were maintained at 1.00 ml/min and 2000 psi respectively. The injection volume and detection wavelength were fixed at 20 μl and 224 nm respectively.

Each degraded drug solution was diluted up to 10 times with mobile phase rendered as diluent. The acid and alkali hydrolyzed solutions were neutralized before dilution. The solid drug samples exposed to thermal and photolytic conditions were rendered into solutions (1 mg/ml) in a diluent of acetonitrile: DDW (10:90 %, v/v) before final dilution by mobile phase for chromatographic analysis.

3.4.1.7 Analysis of Formulation

Twenty tablets were weighed and finely powdered. An accurately weighed portion of the powder equivalent to 4 mg of CPH was transferred to volumetric flask and acetonitrile: water in ratio of 10:90 (%v/v) was added and sonicated for 10 min. The solution was filtered through Whatman filter paper (No. 42) into 10 ml volumetric flask and then diluted to volume with water to get stock sample solution. (400 µg/ml) Aliquot of 2.5 ml was pipette out from above prepared solution and diluted up to 10 ml with water to get the working sample solution (100 µg/ml). Different volumes of working sample solution were taken and diluted with mobile phase to get different concentrations of CPH. The above prepared solutions were analyzed by HPLC for the content of CPH in formulation.

3.4.1.8 Method validation [20]

The method was validated in accordance with ICH guideline Q2 (R1). The proposed RP-HPLC method showed good linearity in the concentration range of 3-18 µg/ml. Precision was undertaken as Inter-day and intra-day precision which were measured in terms of % RSD. The experiment was repeated 3 times in a day (Intraday precision) and the average % RSD values of the results were calculated. Similarly the experiment was repeated on 3 different days (Inter day precision) and the average % RSD value for absorbance were calculated. The low value of SD obtained confirms the precision of the method. For LOD and LOQ, calibration curve was repeated for 9 times and the standard deviation (SD) of the intercepts was calculated. According to ICH recommendations, the approach based on the standard deviations (S.D) of the y-intercept and the slope was used for determining the LOD and LOQ. Accuracy of the method was confirmed by recovery study from marketed formulation at 3 level of standard addition (80%, 100%, and 120%) of label claim. Recovery greater than 98 % with low SD justified the accuracy of the method. Robustness of the method was determined by making small but deliberate changes in various chromatographic parameters of the optimized method.

3.4.2 Results and discussion

3.4.2.1 Determination of suitable wavelength

The UV spectrum of CPH is presented in Figure 3.2. The spectrum indicates that λmax of CPH is 224 nm. The UV spectra of CPH and its impurities were extracted in PDA detector from 200-400 nm but sufficient absorption of drug as well as DP's was observed 224 nm itself and was used as analytical wavelength.

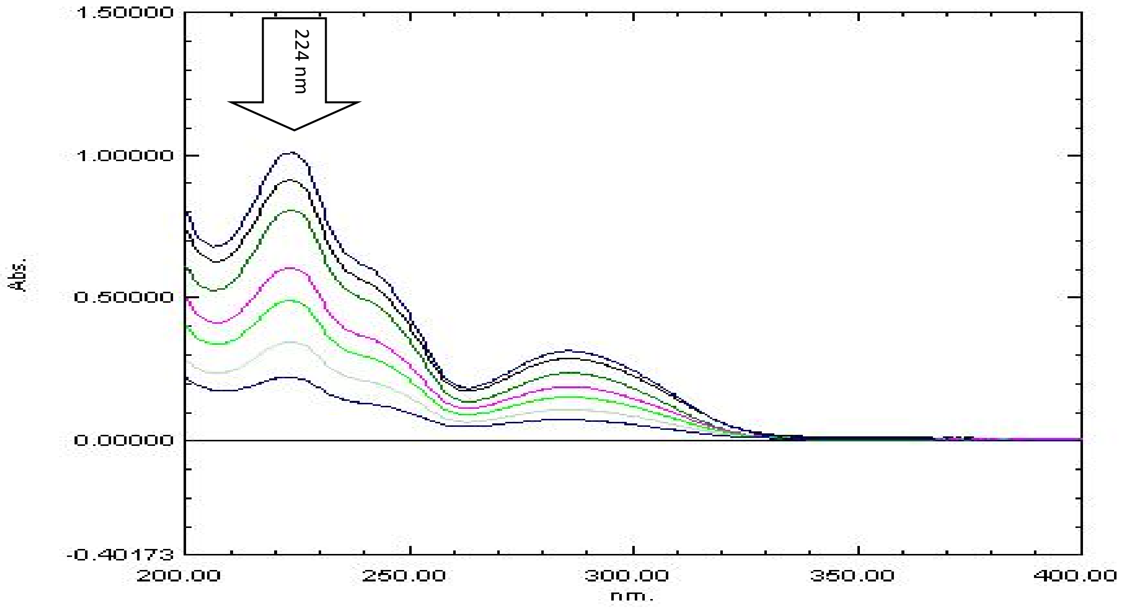


Figure 3.2: UV Spectra of CPH

3.4.2.2 Method optimization and development

To optimize the chromatographic conditions, the effect of chromatographic variables such as composition of mobile phase, ratio of mobile phase, pH of mobile phase, effect of organic modifiers and flow rate were studied as represented in Table 3.1. The resulting chromatograms were recorded and the chromatographic parameters such as retention time, tailing factor, resolution and theoretical plates were calculated. The conditions that gave the best resolution, symmetry and theoretical plate were selected for estimation. Finally, a simple and inexpensive method could be developed by using acetonitrile-methanol-20 mM ammonium formate (pH 5.5 adjusted with 0.2% formic acid, 0.1% TEA (40:10:50, v/v/v) as the mobile phase. The mobile phase flow rate and typical pressure of the system were maintained at 1.00 ml/min and 2000 psi respectively. The optimized chromatographic parameters for the study are represented in Table 3.2. The analysis was performed at ambient temperature with injection volume of 20 µl. The linearity range for analysis of CPH was obtained as 3-18 µg/ml. The chromatograms and calibration curve of CPH are shown in Figure 3.3 and Figure 3.4 respectively. The details of system suitability parameters for the developed method are represented in Table 3.3.

Table 3.1: Mobile phase optimization trials

Mobile phase	Ratio (%v/v/v)	Column	Flow rate (ml/min)	CPH	
				Rt (min)	Peak shape
methanol: water	50:50	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	9.0	Asymmetic peak
methanol: water	60:40	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	9.4	Broad peak
methanol: water	40:60	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	8.4	Broad peak
ammonium acetate buffer 20mM :ACN(pH =4)	60:40	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	7.5	Broad peak
ammonium acetate buffer 20 mM :ACN:methanol(pH=4)	70:30	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	6.9	Split peak
ammonium acetate buffer 20 mM :ACN (pH=3)	40:60	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	10.4	Broad peak
ammonium formate buffer 20 mM:ACN:methanol (pH=5.5)	60:30: 10	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	4.1	Broad peak
ammonium formatebuffer 20 mM:ACN:methanol (pH=5.5)	40:50: 30	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	4.4	Symmetric peak

ammonium formatebuffer 20 mM :ACN (pH=5.0)	50:50	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	8.4	Broad peak
ammonium formatebuffer 20 mM :ACN (pH=4.5)	60:40	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	7.5	Split peak
ammonium formatebuffer 20 mM :ACN (pH=5.5)	60:40	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	6.8	Symmetric peak
ammonium formatebuffer 20 mM :ACN:methanol (pH=5.5),0.1% TEA	**50:40: 10**	**BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)**	**1.0**	**6.8**	**Sharp and Symmetric peak**

Table 3.2 Optimized chromatographic parameters for analysis of CPH

Method parameter	**Optimized value**
Column	BDS hypersil c-18 (250 mm x 4.6 mm, 5 µm)
Mobile phase	ammonium formate buffer 20mM :ACN:methanol(pH=5.5), 0.1%TEA 50:40:10
Retention time	6.8 min
λmax	224 nm
Flow rate	1 ml/min
Temperature	Ambient

Figure 3.3 Optimized chromatogram of CPH in range of 3-18 μg/ml

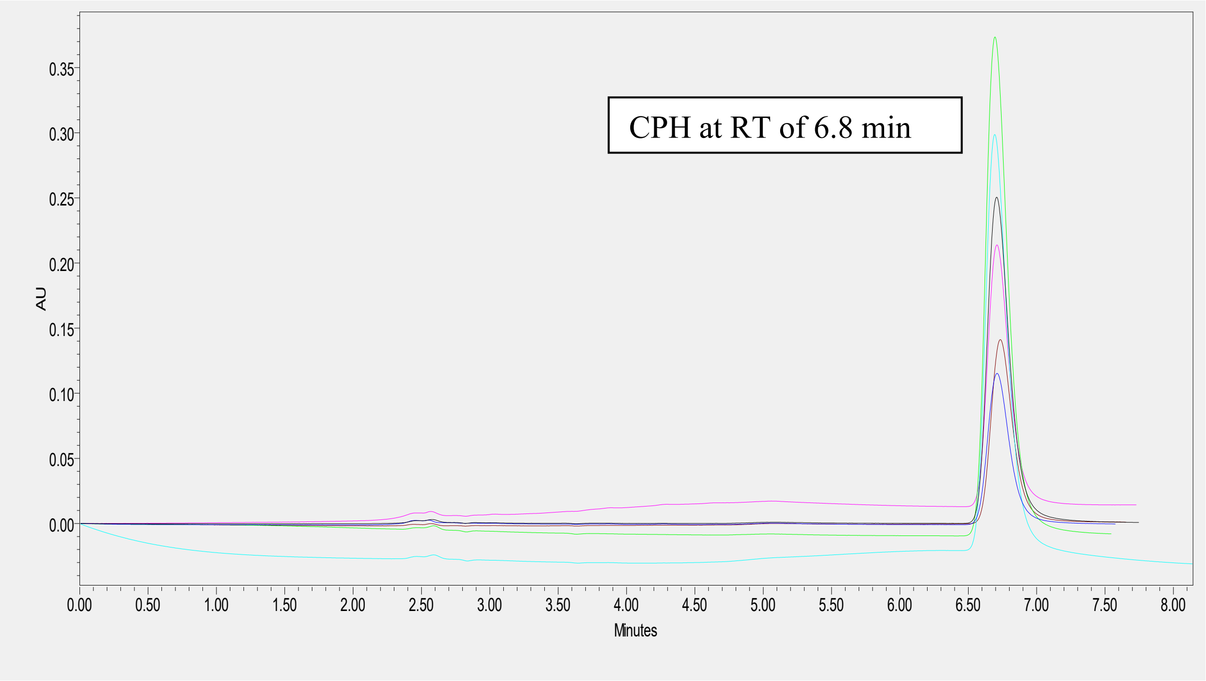


Figure 3.4 Calibration curve of CPH by optimized HPLC-PDA method

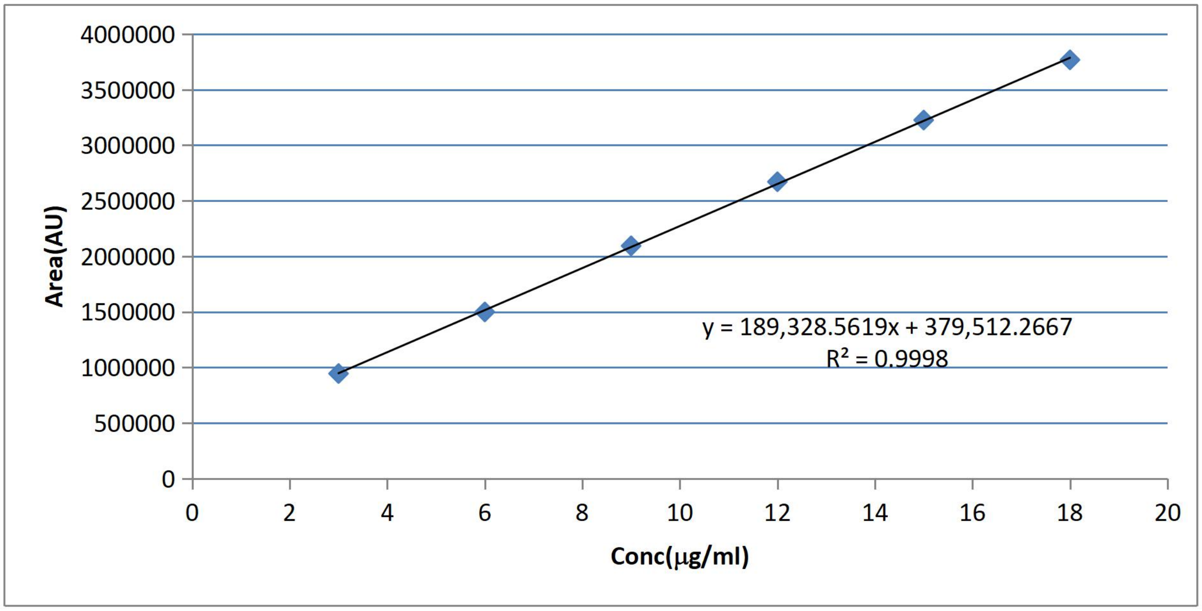


Table 3.3 Summary of system suitability parameters (*Data obtained from 6 replicate injections)

Parameters	**Data obtained* (CPH)**
Retention time (min) ± SD	6.8 ± 0.07
Theoretical plate ± SD	5435± 67.89
Tailing factor ± SD	1.21 ± 0.01

*Data obtained from 6 replicate injections

3.4.2.3 Method validation using ICH Q2 (R1) guideline

The validation of developed HPLC-PDA method was done as per ICH guidelines. The summary of validation parameters is represented in Table 3.4.

The linearity of the HPLC detector response for determination of CPH was evaluated by analyzing a series of concentrations of compound. The calibration range was established with respect to the practical range necessary, according to the marketed formulation, to give accurate, precise and linear results.

The precision was performed at three concentration levels for compound in triplicate. The three levels were 3, 9, 18 µg/ml of CPH. The experiment was repeated three times in a day for intra-day precision and on three different days for inter-day precision.

The accuracy was performed by standard addition method. Known amounts of CPH were added to a known concentration of the commercial tablet formulation at three levels of standard addition (80%, 100%, and 120%). As discussed in section 3.4.1.7, from the working stock solution of formulation, 1 ml each of 50 µg/ml was transferred to 9 different 10 ml volumetric flasks. To it 40 µg/ml (80%), 50 µg/ml (100%), 60 µg/ml (120%) of standard CPH was added in 3 different flasks in triplicate and diluted up to mark giving final concentrations 9, 10, 11 µg/ml respectively in triplicate. The resulting mixtures were analyzed and the percentage recovery was calculated. (Table 3.5)

For determining the limit of detection (LOD) and limit of quantitation (LOQ), the approach based on the standard deviation (S.D) of the y-intercept and the slope was used and the values were obtained.

Robustness study was performed by making small but deliberate changes in flow rate by ±0.1 (0.9 mL/min, 1.0 mL/min, 1.1 mL/min) and change in the composition of Organic solution (acetonitrile) by ±2% (38%, 40% and 42%) and variation in detection wavelength) by ±2% (222, 224, 226 nm) as represented in Table 3.6. From these results it is concluded that the method has capacity to withstand some extent of human or system errors.

The stability of the working stock solutions was studied at room temperature and in refrigerator (8-25ºC) condition for 24 hours. No additional peaks were found on the chromatogram indicating the stability of CPH sample solution.

The specificity of the method was assessed by analyzing the formulation, indicating no interference of the excipients on the developed method. (Table 3.9) Also a peak purity study verifies the specificity of developed method. (Table 3.7)
For system suitability study, six replicates of the solution 4 µg/ml CPH were used and their %RSD was found. (Table 3.3)

Table 3.4 Summary of validation parameters

Parameters	**CPH**
Analytical wavelength(nm)	224nm
Retention time (min)	6.8 ± 0.07 min
Linearity range (µg/ml)	3-18
Regression equation	Y = 189,328.5619x + 379,512.2667
Correlation coefficient	0.9998
Intraday precision (%RSD)	0.24
Inter day precision (%RSD)	0.71
LOD (µg/ml)	0.13
LOQ (µg/ml)	0.39

Table 3.5 Recovery studies from formulation [Heptidin tablets by Unison pharmaceuticals Pvt. ltd. (4 mg)]

Excess drug added to analyte (%)	**Theoretical content (µg/ml)**	***Amount recovered (µg/ml)**	**%Recovery±SD**
0	5	4.99	99.74±0.19
80	9	4.89	98.87±0.08
100	10	4.95	99.45±0.21
120	11	4.90	99.10±0.05

*Average of three determinations

Table 3.6 Robustness study for developed method

Factor	**Retention time (min) CPH**	**Peak area (AU)**
A. Flow rate (ml/min)		
0.9	7.06	944525
1	6.82	944560

1.1	6.59	944578
Mean ±SD	6.82±0.10	944554±524
B. Ratio of ACN:		
38 %	6.91	945236
40%	6.81	944369
42%	6.85	944799
Mean ±SD	6.85±0.09	944801±857
C. Wavelength		
222 nm	6.90	944109
224 nm	6.80	944896
226nm	6.90	944789
Mean ±SD	6.87±0.08	944598±703

3.4.2.4 Stress degradation studies [17, 18, and 19]

Stress degradation study was undertaken for hydrolysis (acid, base, neutral), thermal conditions, photolytic conditions and oxidation conditions. The summary of the study is shown in table 3.8. Degradation obtained was calculated using the formula as follows:

$$\%\text{Degradation} = \frac{\text{Initial area of untreated stock solution} - \text{reduced area of treated stock solution}}{\text{Actual area of untreated stock solution}} \text{X}100$$

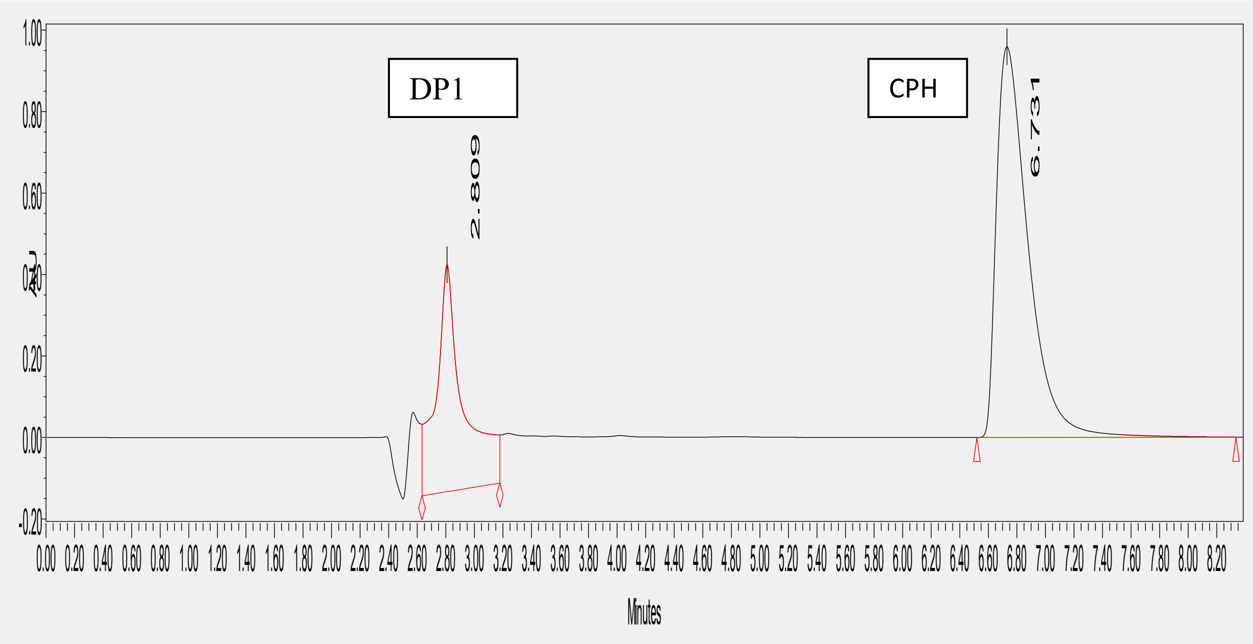
CPH on stress degradation yielded two degradation products i.e., DP1and DP2. DP1 was obtained by acid hydrolysis at retention time of 2.80 min in a HPLC chromatogram. (Figure. 3.5a) Degradation product 2 (DP2) was obtained by base hydrolysis at retention time of 2.80 min in a HPLC chromatogram. (Figure. 3.5b) The drug was also subjected to oxidation condition by applying 6% H_2O_2 at 100°C for up to 12 hours but no degradation product was observed. However, a decrease in area was observed as compared to standard and also aliquots taken at different time intervals viz., 3, 6 hours demonstrated a decrease in area with respect to time with similar stressor conditions. The drug was also subjected to neutral hydrolysis condition by applying temperature of 100°C for up to 5 hours but no degradation product was observed. However, a decrease in area was observed as compared to standard and also aliquots taken at different time intervals viz., 1, 3 hours demonstrated a decrease in area with respect to time with similar stressor conditions. The drug was also subjected to thermal degradation by applying dry heat condition on solid CPH 100 mg spreaded on glass petridish as a thin layer about 1mm thickness in a high precision oven at 60°C for up to 10 days but no degradation product was observed. However, a decrease in area was observed as compared to standard and also aliquots taken at different time intervals viz., 1, 5 days demonstrated a decrease in area with respect to time with similar stressor conditions. The drug was also subjected to photolytic degradation by applying dry UV light exposure consisting of total UV light of about 144 Wh/m^2 and 5382 Lux on solid CPH 100 mg spreaded on glass petridish as a thin layer about 1mm thickness in the photo stability chamber for up to 15 days but no degradation product was observed. However, a decrease in area was observed as compared to standard and also aliquots taken at different time intervals viz., 1,3,5,7 days demonstrated a decrease in area with respect to time with similar stressor conditions (Table 3.8). All the degradation samples were suitably diluted with mobile phase to make final concentration of 100 μg/ml with respect to CPH and filtered through 0.2 μm nylon membrane syringe filter prior to injection in HPLC.

As per the literature survey, recently stability indicating derivative spectroflorimetric [28] and HPLC method with florescence detector [25] was reported during the tenure of our study. In both studies an oxidative degradation product was observed. The reported method was not LC/MS/MS compatible and the acid and base hydrolysis degradation product eluted by our LC/MS/MS compatible chromatographic method was not observed in the reported method. Inspite of many changes in polarity by mobile phase variations, although drug degradation was observed for oxidative conditions, no degradation product was detected by our chromatographic method. The methods [25, 28] reporting the oxidative impurity are based on florescence detection which has normally much more sensitivity for detection. Possibly the amount of oxidative impurity generated was beyond the detection levels of PDA detector. Also they [25] have used much high concentration of stressor for oxidative degradation (i.e., 15% H_2O_2) which also may be the reason for detection of impurity in their studies contrary to our stability studies. Similarly as apparent from previous studies CPH does not degrade in mild stress conditions, strong stressor conditions are needed for degradation studies of CPH. On application of acid and base hydrolysis stressor conditions two UV visible degradation products viz., DP1 and DP2 were obtained respectively. Whereas for oxidative, neutral hydrolysis, dry heat, thermal and photolytic conditions, instead of drug degradation, we have not obtained UV-visible degradation products. Many changes in the polarity of mobile phase were done with long run times still no degradation products were visible for stress conditions other than acid and base hydrolysis conditions. Also influence of column parameters was monitored by making changes in column type and column characteristics used for the study, but still only DP1 and DP2 were obtained for our stress degradation studies. Figure 3.6 represents the HPLC chromatogram under all stressor conditions.

Hence, based on above results, the drug is found susceptible to all stressor conditions, but gives a UV visible and detectable degradation product in acid and alkali conditions only when run on our optimized chromatographic conditions. Peak purity data is shown in Table 3.7.

Figure 3.5 HPLC chromatogram of CPH and DP's

a) By acid hydrolysis



b) By alkali hydrolysis

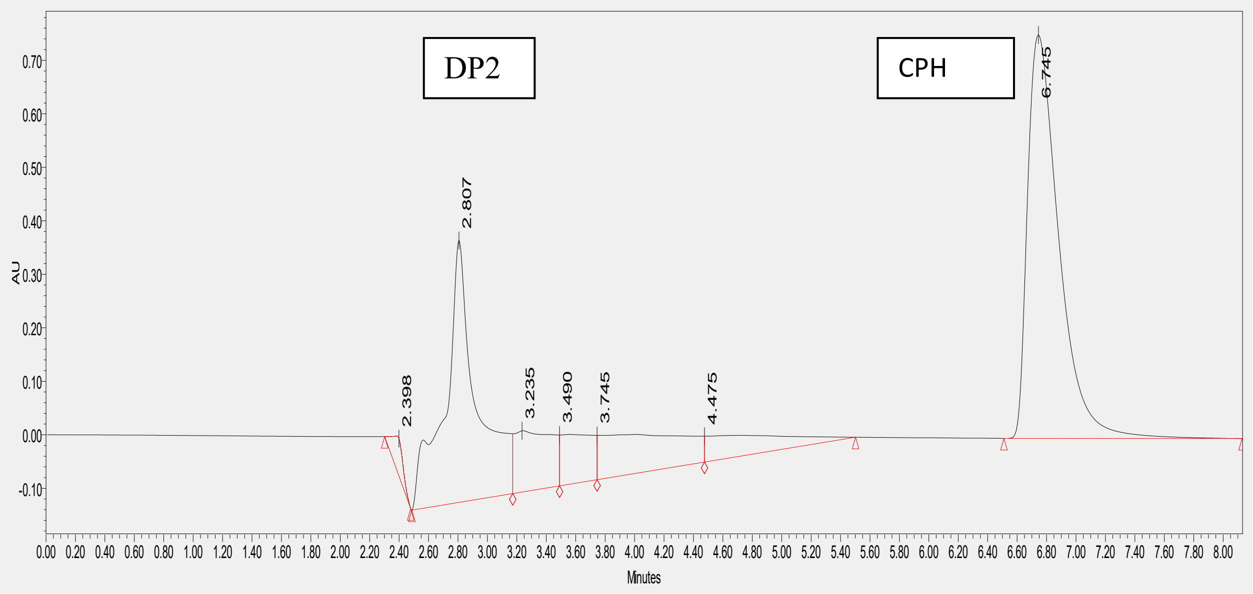
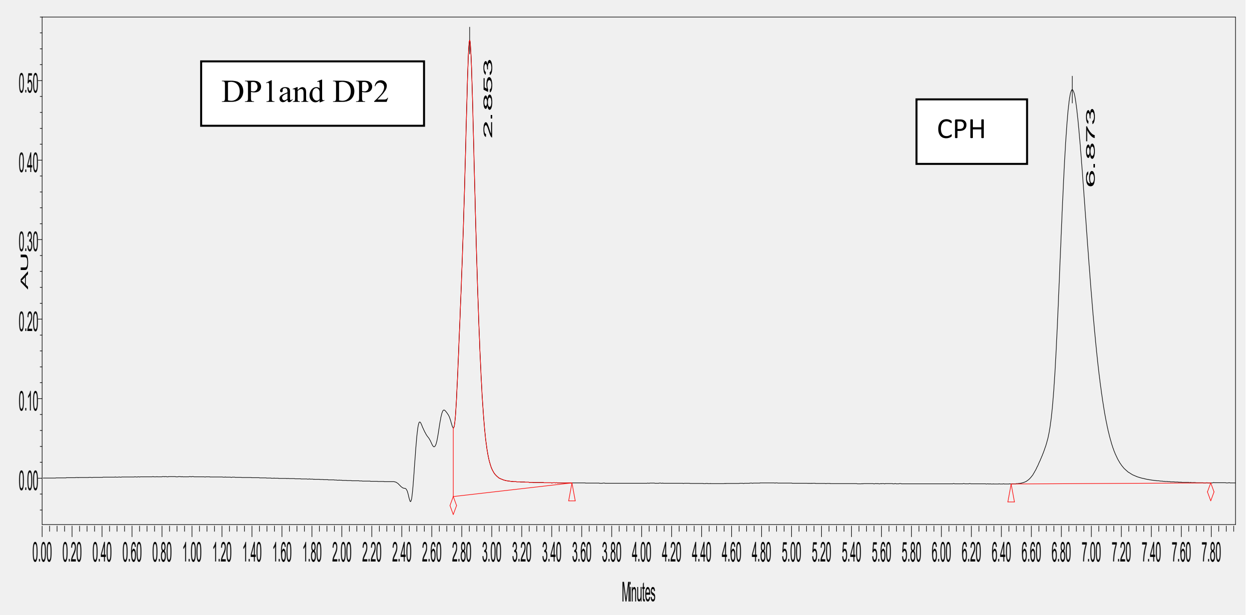


Figure 3.6 HPLC chromatogram under all stressor conditions



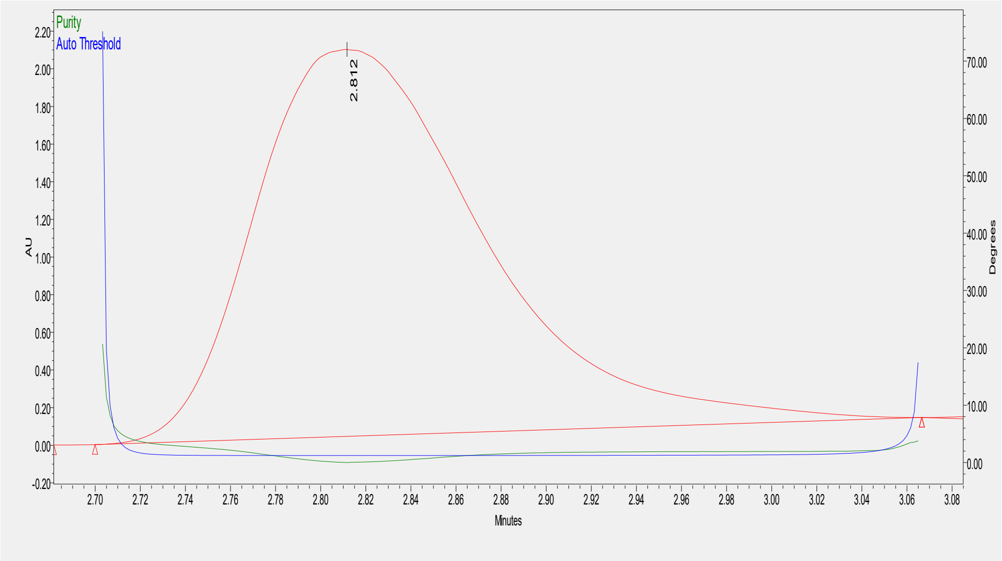
3.4.2.5 Peak purity studies

Peak purity studies were done at various stages during optimization of method. The data for peak purity studies at various stages is shown in Table 3.7. When chromatograms were taken in individual stressor conditions viz., for acid hydrolysis and base hydrolysis, pure peaks of standard drug as well as DP's were observed. (Figure 3.7)

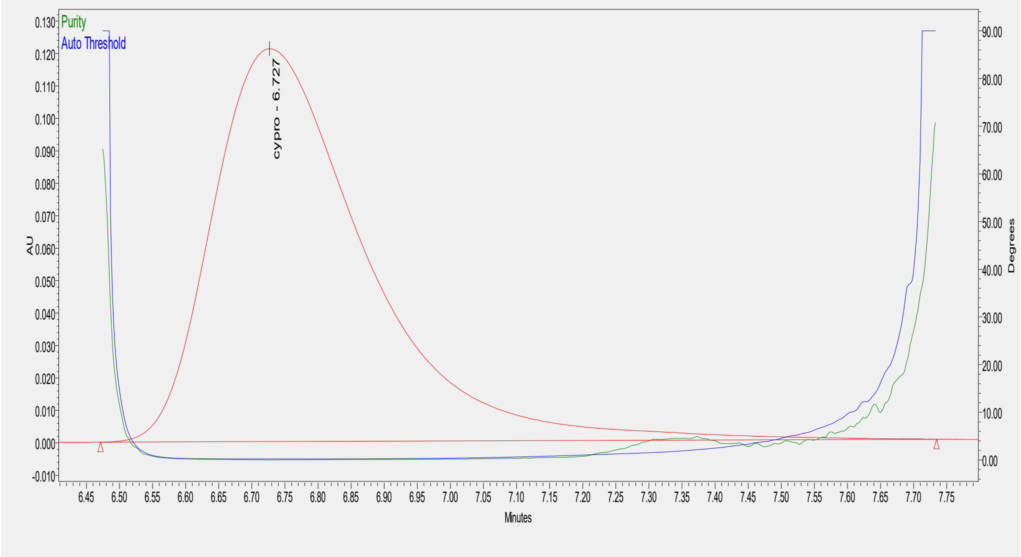
Whereas when the chromatogram of a sample containing all the DP's was taken, it was observed that the peak of standard drug was pure whereas the peak at RT of 2.8 min was not pure as at RT of 2.8 min, the DP1 of acid stressor condition as well as DP2 of base stressor condition are eluted. To check if there are any co-eluting species at RT of 2.8 min, further investigation was done for peak purity analysis. As, we already know PDA detector plots spectra for each second and at each wavelength fed in generated method (generally 200-400 nm) for the peak of our study. The purity angle is calculated by checking the homogeneity obtained from spectral data for every component lying in between the peak width whereas purity threshold is calculated from the area of chromatogram where no peak is getting eluted for checking the interference from noise, solvent and other parameters. Now while calculating if we enable peak purity using the auto threshold option from the system, the purity threshold is calculated from baseline taking AU value -0.05 Y-axis and we get purity threshold value to be 1.069 and purity angle to be 3.708, but if we calculate peak purity manually using Noise+solvent option for calculating peak purity, which may occur while chromatographic analysis, purity threshold is calculated from baseline taking AU value -0.01 on Y-axis we get purity threshold to be 10.323 and purity angle to be 3.612 signifying peak to be pure. The solvent angle selected while calculating peak purity by manual method using Noise+solvent option was 1, which was lower than the purity angle of DP1 as well DP2 while separate stress conditions applied in acid as well as base hydrolysis condition as well for isolated DP1, thus confirming the calculation procedure for peak purity to be authentic. Thus, we can say that peak is spectrally pure. Further, LC/MS/MS studies also confirm similar m/z values for DP1 and DP2 assuring similar chemical spectra characteristics for DP1 and DP2.

Figure 3.7 Peak purity studies

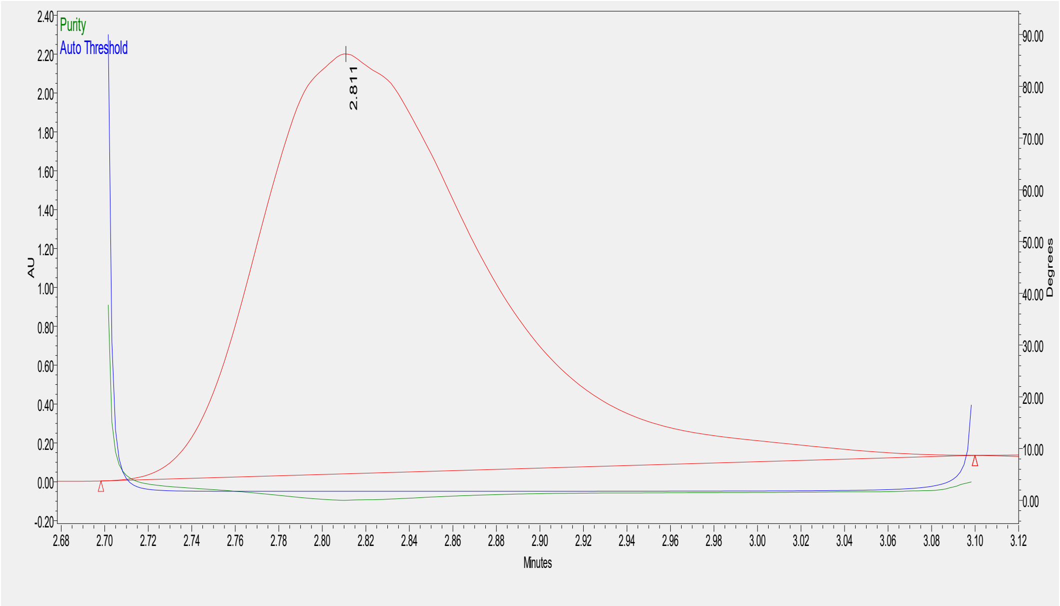
a) **Peak purity of DP1 on acid hydrolysis stressor condition**



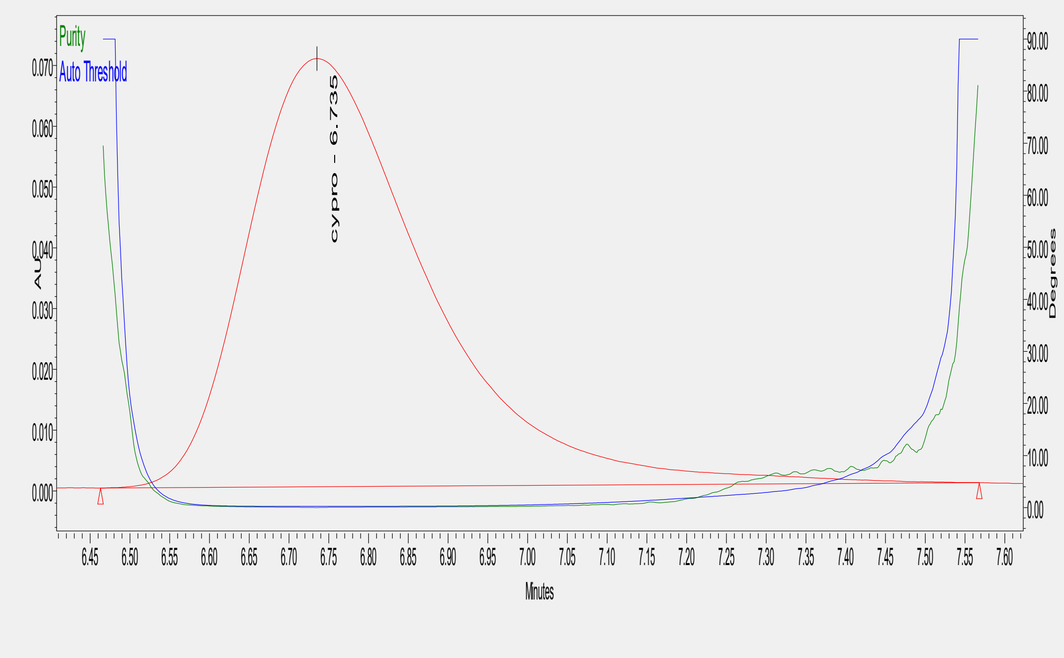
b) **Peak purity of CPH on acid hydrolysis stressor condition**



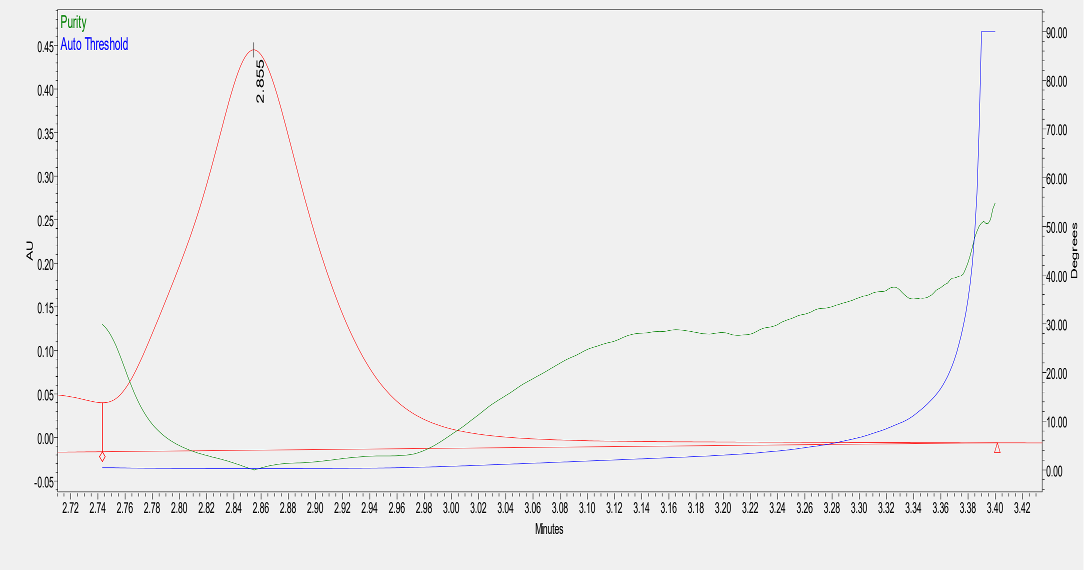
c) Peak purity of DP2 on base hydrolysis stressor condition



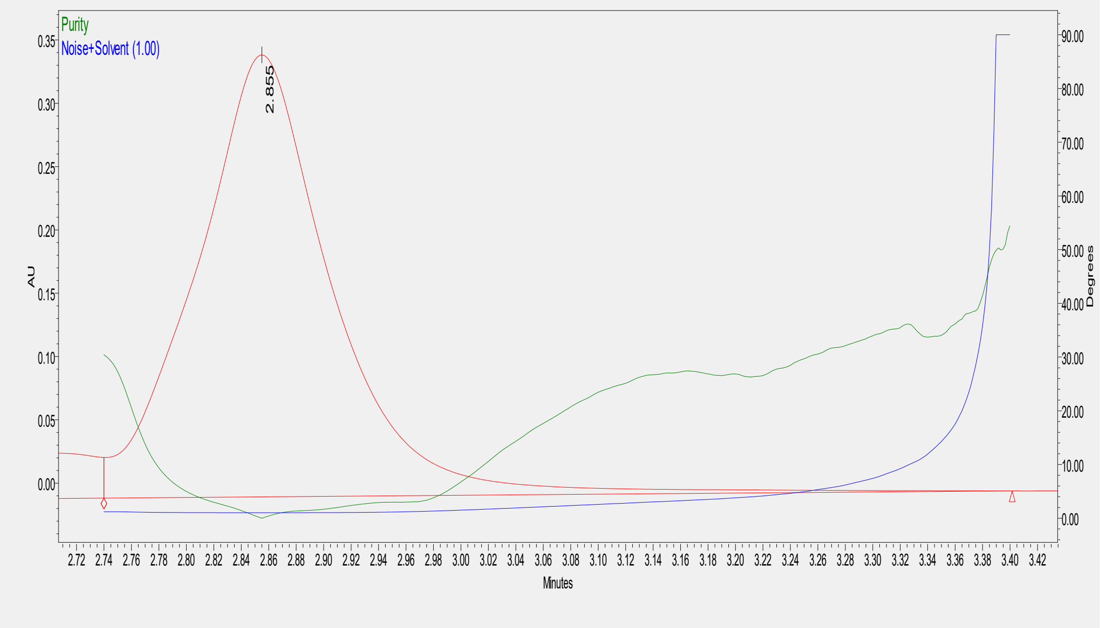
d) Peak purity of CPH on base hydrolysis stressor condition



e) Peak purity of DP1 and DP2 under all stressor conditions (Auto threshold)



f) Peak purity of DP1 and DP2 under all stressor conditions (Noise+solvent)



g) Peak purity of CPH under all stressor conditions

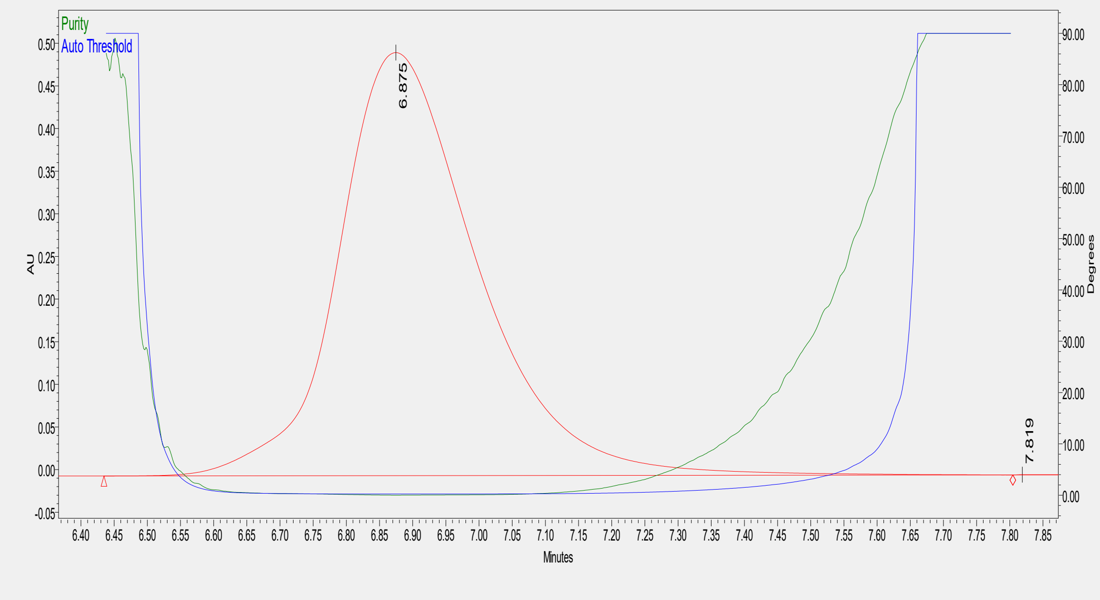


Table 3.7: Peak purity studies

Sr.No	Peak	Peak purity angle	Peak purity threshold
1	DP1 in acid hydrolysis stressor condition	1.124	1.232
2	CPH on acid hydrolysis stressor condition	0.709	0.880
3	DP2 on base hydrolysis stressor condition	1.264	1.459
4	CPH on base hydrolysis stressor condition	0.923	1.174
5	DP1 and DP2 under all stressor conditions (Auto threshold)	3.708	1.069
5	DP1 and DP2 under all stressor conditions (Noise+solvent)	3.612	10.323
6	CPH under all stressor conditions	0.253	0.255

Table 3.8 Summary of stress degradation conditions applied to CPH

Sr.No	Stressor condition	% Degradation (API)	% Degradation (Formulation)	RT of DP
1	Acid hydrolysis	11.31	12.61	2.8 min (DP1)
2	Base hydrolysis	15.53	15.71	2.8 min (DP2)
3	Neutral hydrolysis	4.71	4.93	No DP observed
4	Oxidation	21.68	22.51	No DP observed
5	Photolytic	3.13	4.78	No DP observed
6	Thermal	6.75	6.90	No DP observed

3.4.2.6 Applicability of method

The developed method was successfully applied for estimation of CPH in its commercial formulation [Heptidin tablets by Unison pharmaceuticals pvt ltd. (4 mg)]. The % assay 100.404±0.04 (CPH±SD), the label claim was 4mg/ml as represented in table 3.9. The developed method was also used to analyze stress degraded samples of the same CPH formulation. Stress degradation were carried out under same conditions as specified for

API and analyzed in the similar way using same chromatographic conditions. As presented in table 3.8 minor variations were observed in degradation of API and formulation.

Table 3.9 Applicability of method

Actual conc. (mg in 10 ml)	Amount of CPH found (mg in 10 ml)	%Label claim	Standard Deviation	%RSD
4	3.99	99.87%	0.04	0.95 %
4	3.98	99.65%		
4	4.07	101.95%		
4	4.04	101.22%		
4	3.98	99.67%		
4	4.02	100.05%		

3.5 SECTION-B

ISOLATION AND CHARACTERIZATION OF MAJOR DEGRADATION PRODUCT OF CPH

Forced degradation studies implicated 1 degradation product each in acid and base condition, but as per LC/MS/MS analysis (Figure 3.17), they were inferred to be similar degradation products, thus isolation of only DP1 was carried forward. DP1 was formed as a major degradation product and it was isolated through conventional thin layer chromatography incorporating preparative technique using precoated silica gel plates for isolation. For its generation, CPH (1.0 g) in 3 M HCl in a 100ml RBF was refluxed at 100°C for 5 hours in an oilbath. The solution was cooled to room temperature, neutralized with 3 M NaOH solution. The sample thus formed was used for isolation using a precoated silica gel TLC plate acquired from Spectrochem.

3.5.1 Experimental

3.5.1.1 Equipments and Chromatographic Conditions (TLC, LC/MS/MS, NMR, FTIR and DSC conditions)

The preparative TLC was performed on precoated silica gel plates from Merck and 20cm X 20 cm (length X diameter) twin trough glass chamber.

For LC/MS/MS studies a LCQ fleet, Thermo Fisher scientific instrument coupled with quaternary system delivery module in positive and negative ESI (electro spray ionization) mode was used. The nebulizer pressure was set at 20 psi using nitrogen gas; the gas temperature was set at 2500C using drying gas nitrogen at 30 psi pressure and capillary voltage 5500 V. The source parameters like Sheath gas flow rate, Aux gas flow rate, Capillary temperature, Ion transfer tube temperature, Discharge current, Vaporizing temperature were optimized at 50 Arb, 30 Arb, 270°C, 4 MA and 380°C respectively. For data acquisition and processing Xcalibur software was used.

For FTIR studies IR-Affinity Fourier Transform infrared (FTIR) spectrometer of Shimadzu was used equipped with IR solution software. The samples were analyzed by Miracle 10 single reflection attenuated total reflectance accessory.

NMR Spectroscopy analysis (^{1}H NMR, ^{13}C NMR, ^{135}DEPT NMR, D_20 exchange spectra) of CPH and its degradation product I were recorded by using Bruker Advance II 400 NMR spectrometer that consisted of dual broad band probe and z-axis gradients.

The spectra were recorded using DMSO as a solvent and tetramethylsilane as an internal standard. DSC analysis was carried out using DSC60 instrument of Shimadzu equipped with TG60 software.

Other equipments and chromatographic conditions used to check % degradation and purity of isolated degradation products (DPs) were same as described in section 3.4.1.2.

3.5.1.2 Structural characterization of isolated degradation products (DP's)

The probable structure of isolated DPs were proposed based on LC-MS/MS, FT-IR and NMR studies

3.5.2 Results and discussion

3.5.2.1 Isolation of impurity, Data interpretation and characterization by LC/MS/MS, NMR, IR and DSC study [22]

LC/MS/MS study of both degradation products was done along with CPH using the optimized mobile phase. ESI-MS-MS spectra of CPH salt having molar mass 324 gm/mol ionizes and CP is separated giving molecular ion peak at $[M+H]^+$ 288 gm/mol in positive ion mode at retention time of 6.8 min. MS spectra of DP1 was obtained at $[M+H]^+$ 239 gm/mol in positive ion mode at retention time of 2.6 min under acidic stress conditions. MS spectra of DP2 were obtained at $[M+H]^+$ 238.67 gm/mol in positive ion mode at retention time of 2.5 min under alkaline stress conditions. (Figure 3.12) The MS spectra of DP1 as well as DP2 was 85 m/z value less than the parent peak signifying breakage of piperidine ring at from the parent drug in both DP's. Based on above stated results, it was inferred that the similar degradation product is being formed in both acidic as well as alkaline conditions viz., DP1and DP2 are identical and thus further isolation and characterization was done only for DP1.

Preparative HPLC would have given faster results but as the facility was not available, various methods tried for isolation of degradation product were like Preparative TLC, LLE. Out of them preparative TLC turned out to be better method for isolation of impurity. Various solvents were used for isolation DP1 which includes toluene, dichloromethane, diethyl ether, ethyl acetate, acetone, pyridine, methanol, water, acetic acid etc. Various trials were taken for selection of optimum mobile phase for isolation of impurity formed by stress degradation of CPH. Different ratios of various solvents were used for optimization of mobile phase for TLC as represented in Table 3.10. Finally, a good resolution between CPH and DP1 was obtained with mobile phase ratio of Dichloromethane: toluene: Ethyl acetate: methanol in ratio of 60:10:10:20(%v/v/v/v). The TLC chamber was first saturated for 30 min with the optimized TLC mobile phase. The sample was spotted on the TLC plate and kept in the chamber for running of the TLC. The spots were identified under UV light in a TLC visualize UV chamber using short wavelength UV light of 254 nm. The spots thus visualized were marked by using a pencil. The R_f value of standard CPH was found to be 0.9 and that of DP1 was found to be 0.4. Refer Figure 3.8 for optimized TLC chromatogram. The DP1 fraction was then scraped. Then it was dissolved in acetonitrile and sonicated for 30 min. Then solution containing silica gel and DP1was centrifuged at 5000 rpm for 15 min. The supernatant was taken and acetonitrile was evaporated using Rota evaporator and then it was vacuum dried. White colored DP1 was obtained. The purity of isolated DP1 was analyzed in analytical chromatographic mode (HPLC). Refer figure 3.9 HPLC chromatogram of DP isolated from optimized TLC procedure and figure 3.10 for peak purity studies of DP isolated and purified after optimized TLC procedure. Peak purity studies of isolated DP are shown in Table 3.11. For further confirmation DP1 spiked solution of CPH was analyzed by developed analytical HPLC method. (Figure 3.11) Further for chemical structure characterization, NMR data was obtained. The inferences were also supported by FT-IR and DSC studies.

Table 3.10 Optimization of mobile phase of TLC for isolation of degradation product

Mobile phase	Ratio (%v/v/v)	Stationary phase	Rf (min)	CPH and DP results
methanol: DCM	50:50	Silica gel G (250μm 20x20 cm)	-	Spots did not run

N-hexane: methanol	60:40	Silica gel G (250µm 20x20 cm)	-	Spots run thoroughly and spot did not appear on plate
N-hexane: methanol	40:60	Silica gel G (250µm 20x20 cm)	-	Spots run thoroughly and spot did not appear on plate
methanol: chlorofoam	40:60	Silica gel G (250µm 20x20 cm)	-	Spots did not run
DCM: methanol	90:10	Silica gel G (250µm 20x20 cm)	-	Spots merged
DCM: methanol: toluene	90:5:5	Silica gel G (250µm 20x20 cm)	-	Spots merged
DCM: methanol: toluene	10:85:5	Silica gel G (250µm 20x20 cm)	3.5	Resolved spot obtained but it is not reproducible method
DCM: methanol	10:90	Silica gel G (250µm 20x20 cm)	3.6	Spots did not run
DCM: methanol: ammonia	80:10:10	Silica gel G (250µm 20x20 cm)	3.2	Resolved spot obtained, but it is not reproducible method
DCM: methanol: toluene: Ethylacetate	**60:20: 10:10**	**Silica gel G (250µm 20x20 cm)**	**3.5**	**Resolved spots obtained and it is reproducible method**

Figure 3.8 Optimized TLC plate showing resolved spots of CPH and DPI

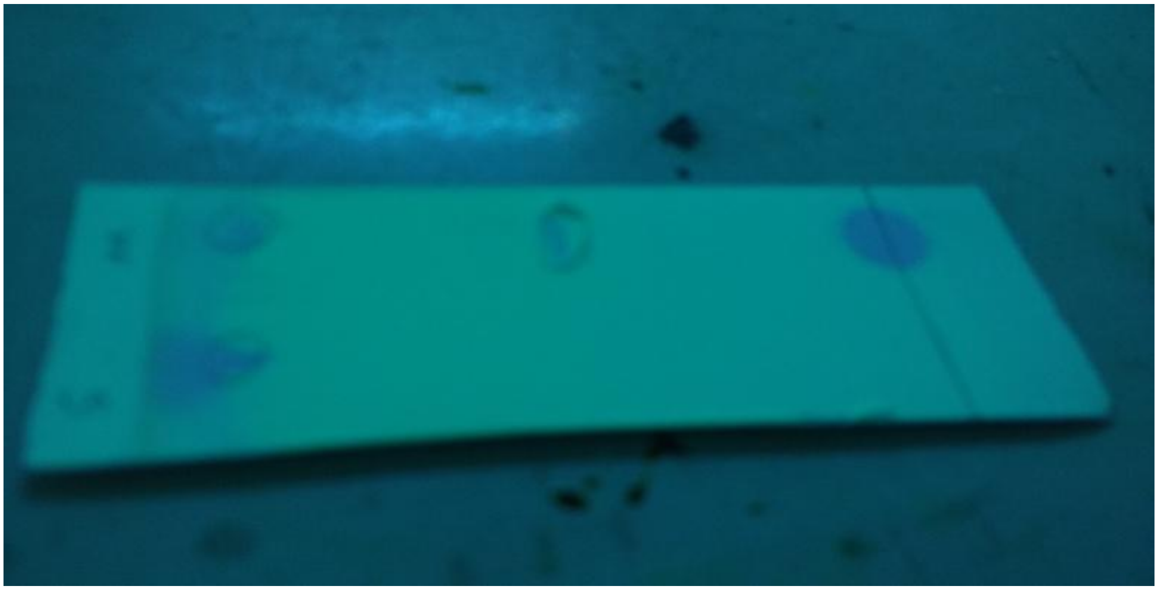


Figure 3.9 HPLC chromatogram of DP isolated from optimized TLC procedure.

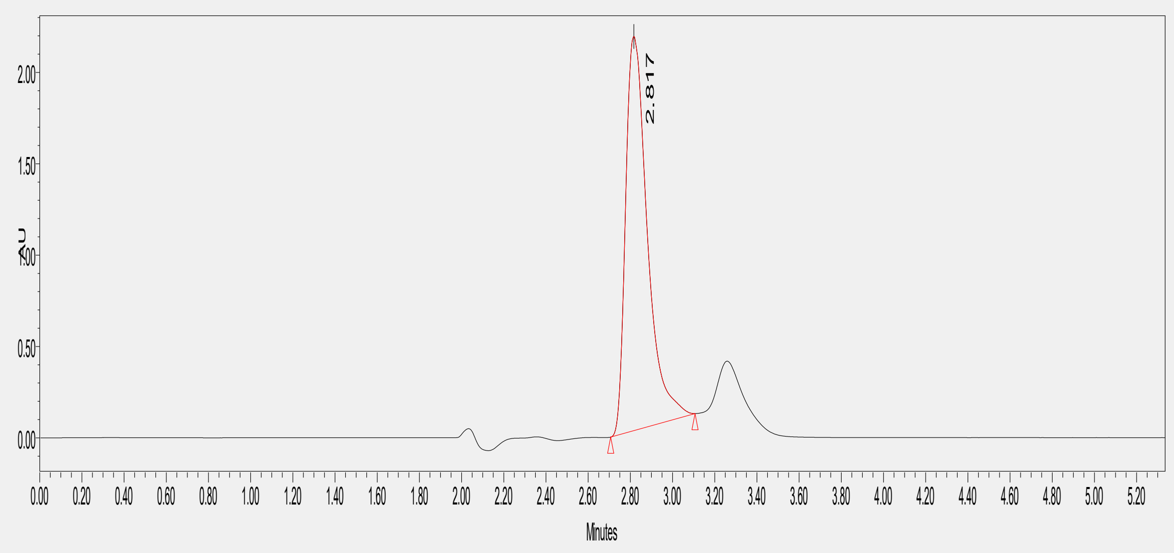


Figure 3.10 Peak purity of DP isolated and purified after optimized TLC procedure

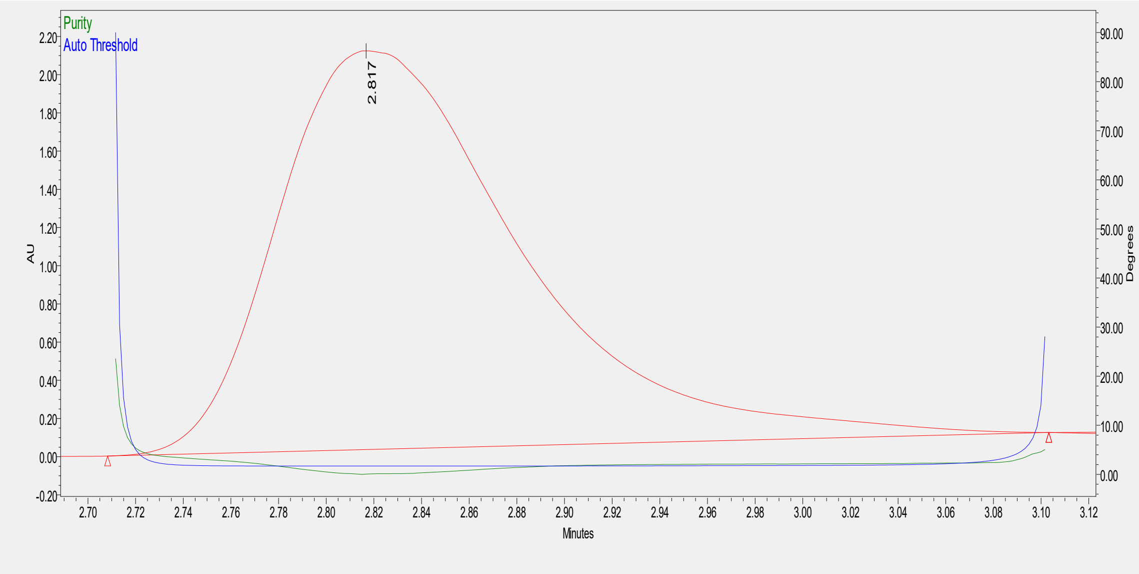


Table 3.11: Peak purity studies for isolated DP

Sr.No	Peak	Peak purity angle	Peak purity threshold
1	DP1 in acid hydrolysis stressor condition	1.676	2.325

Figure 3.11 HPLC chromatogram of isolated DP spiked with standard drug.

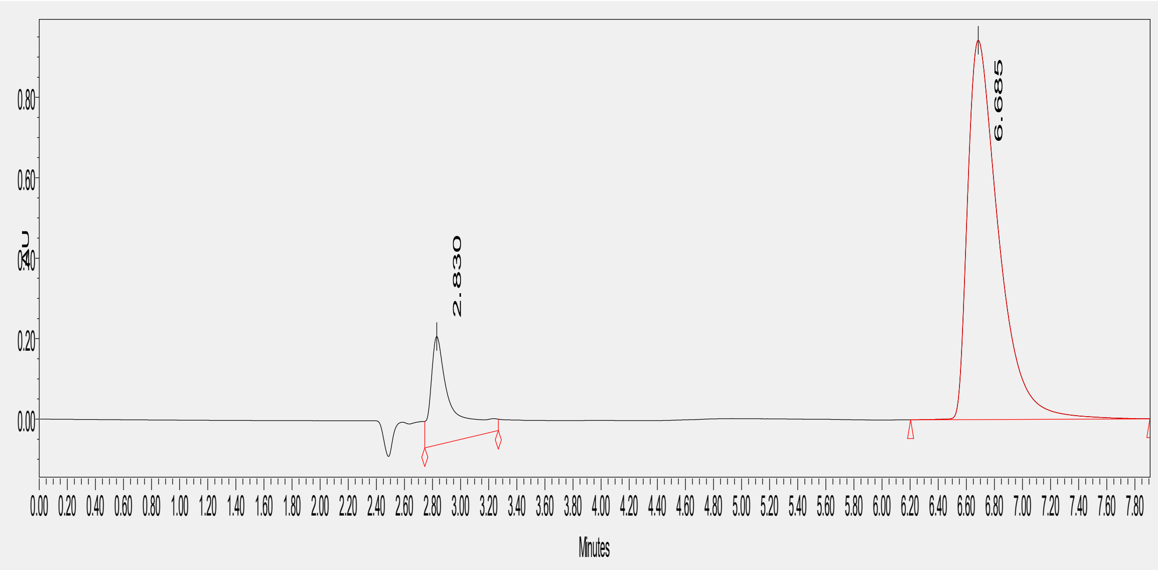
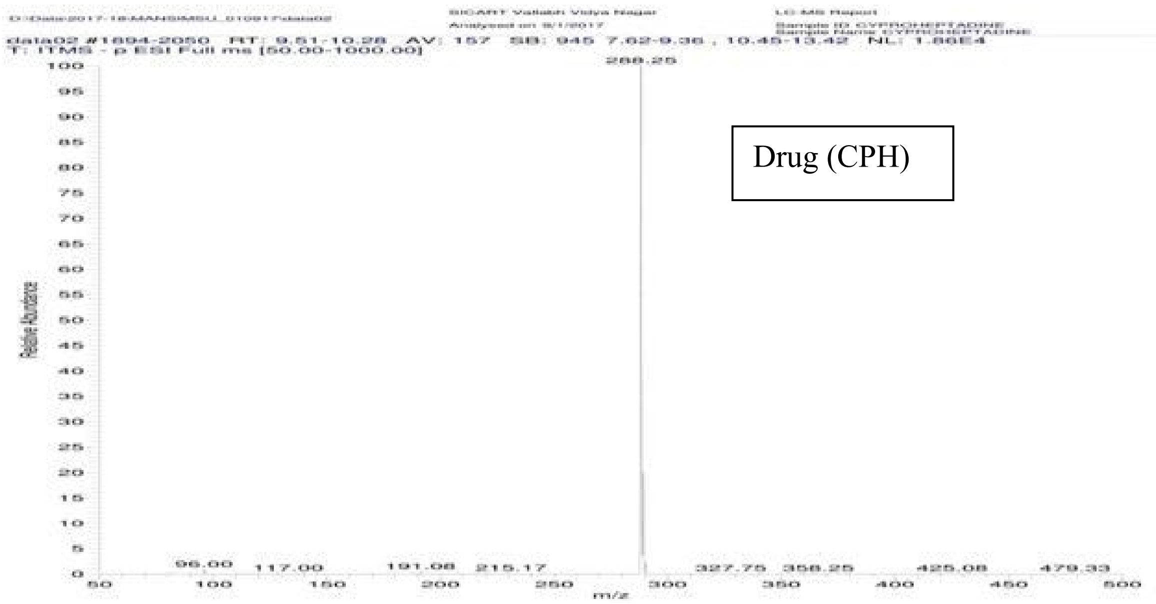
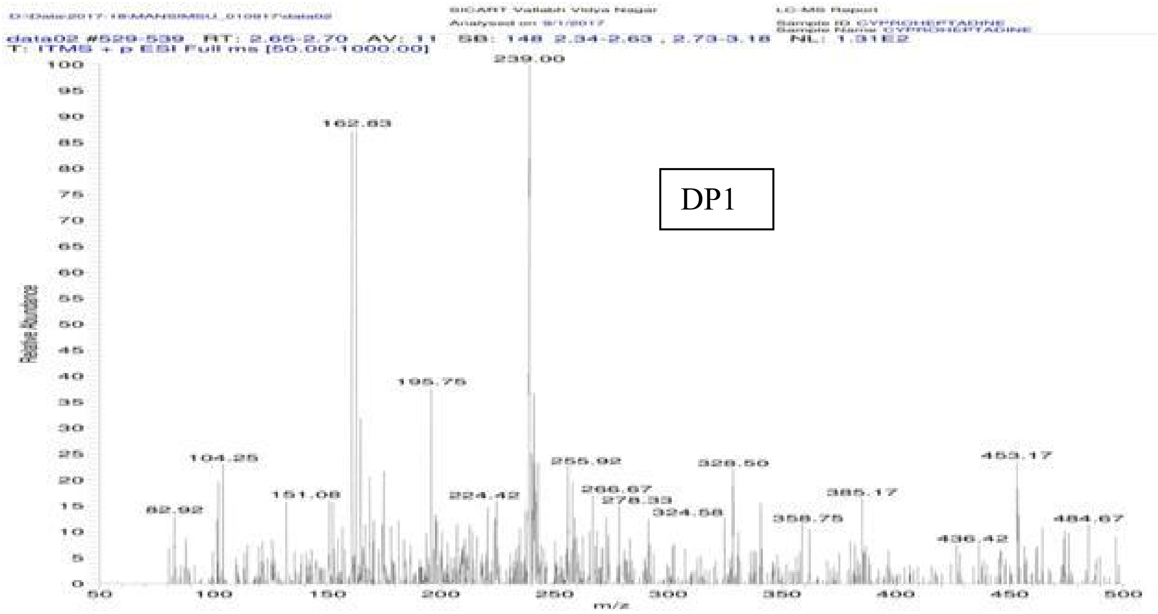


Figure 3.12 LC-MS-MS spectra of stressed samples (a) mass spectral data of standard drug (b) mass spectral data of unknown impurity denoted as DP1 under acidic stress condition (c) mass spectral data of unknown impurity denoted as DP2 under alkaline stress condition

a)



(b)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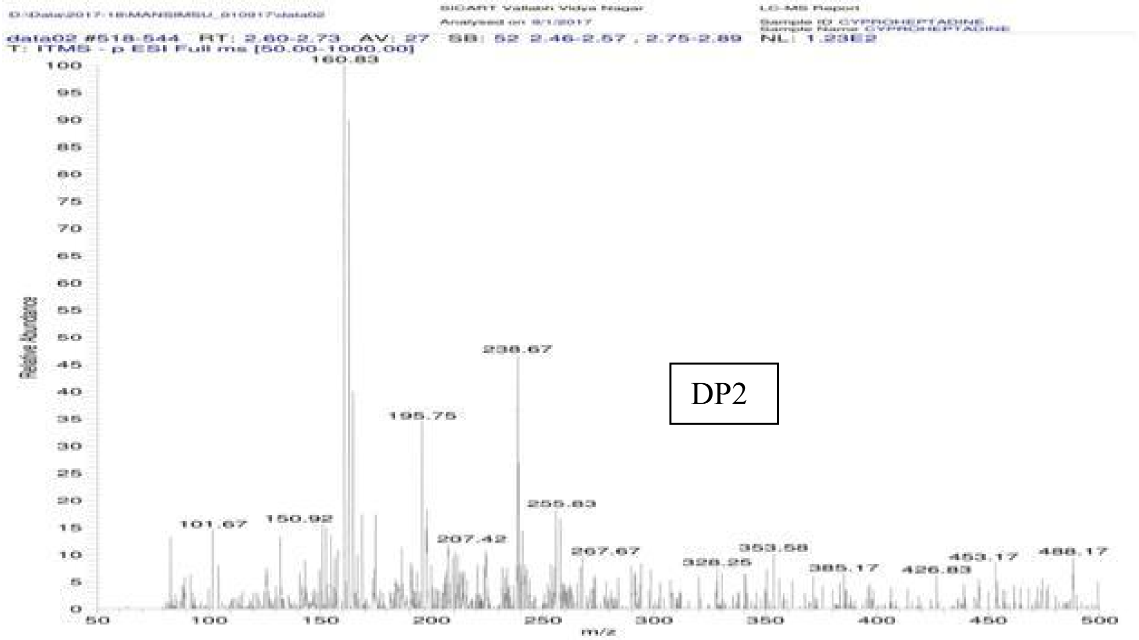
T: ITMS + p ESI Full ms [50.00-1000.00]
DP1
Relative Abundance
m/z
239.00
162.83
195.75
104.25
82.92
151.08
224.42
255.92
266.67
278.33
324.58
328.50
358.75
385.17
436.42
453.17
484.67

(c)


data02 #518-544 RT: 2.60-2.73 AV: 27 SB: 52 2.46-2.57 , 2.75-2.89 NL: 1.23E2
T: ITMS - p ESI Full ms [50.00-1000.00]
DP2
Relative Abundance
m/z
160.83
238.67
195.75
101.67
150.92
207.42
255.83
267.67
328.25
353.58
385.17
426.83
453.17
488.17

LC/MS/MS studies were done to characterize the DP's and to understand the possible degradation pathways.

CPH: The ESI-MS-MS spectrum of CPH showed $[M+H]^+$ ion at 288 m/z in positive mode. The spectrum showed abundant ions at m/z of 288 m/z representing loss of HCl from the parent drug having molar mass of 324 gm/mol. The heaviest peak at m/z of 288 represents the molecular ion peak as well due to presence of abundant ions is also the base peak.

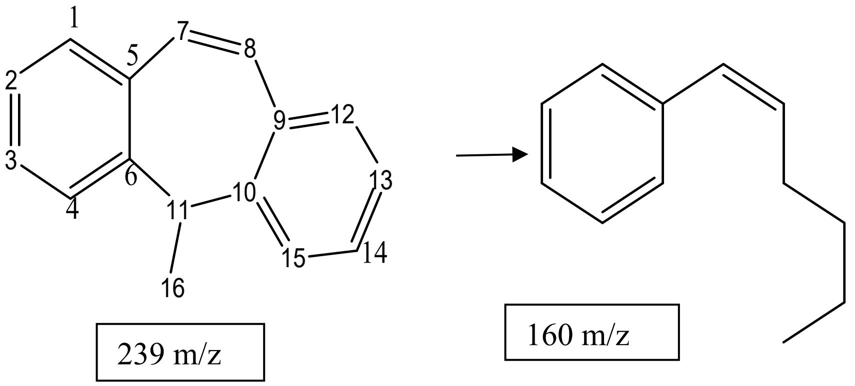
DP1: The ESI-MS-MS spectrum of DP1 showed $[M+H]^+$ ion at 239.00 m/z having the abundant ions as well as the most heavy peak and is thus the molecular ion peak as well as the base peak. The spectrum also showed abundant ions at m/z of 162.63 due to loss of propylene group from the aromatic ring.

DP2: The ESI-MS-MS spectrum of DP2 showed $[M+H]^+$ ion at 238.67 m/z being the molecular ion peak. The spectrum also showed abundant ions at m/z of 160.83 due to loss of propylene group from the aromatic ring. The peak at m/z of 160.83 is having the most abundant ions and is thus the base peak for this spectrum.

Comparison between DP1 and DP2

The fragmentation found in both DP's showed similar pattern. The only difference found was the base peak in DP1 was at 239 m/z whereas in DP2 it was at 160.83 m/z. Due to difference in stressor conditions for DP1 the stress condition was acid hydrolysis whereas for DP2 the stress condition was base hydrolysis, the occurrence of abundant ions for both conditions have varied although the DP formed is similar in both conditions as apparent from similar molecular ion peak and similar fragmentation pattern formed in both conditions.

Fragmentation pattern of DP1 and DP2



The H^1 NMR data of CPH and DP1 were obtained for the structural elucidation. DMSO-d_6 was used as a solvent for analysis. The peak around 3.5 δ ppm signifies DMSO solvent peak. The absence of (s) due to N-CH_3 and a triplet due to CH_2 of piperidine ring probably signifies breakage of 1-methyl piperidine ring from the CPH structure. The C^{13} NMR spectra of CPH and DP1 were also performed for the structural elucidation. The peak around 38 - 42 δ ppm signifies DMSO solvent peak. The presence of 12 identical carbons in CPH whereas presence of only 9 identical carbons in DP1 signifies breakage of 1-methyl piperidine ring from the CPH structure. The (Distortion less enhancement by polarization transfer) $DEPT^{135}$ NMR spectra of CPH and DP1 were also performed for the structural elucidation. The peak around 42 δ ppm signifies DMSO solvent peak. The absence of tertiary carbons corresponding to CH_2 of piperidine ring and primary carbons corresponding to N- CH_3 in DP1 signifies breakage of 1-methyl piperidine ring from the CPH structure. Also the presence of primary carbons due to presence of CH_3 at position number 16.verifies the formation of DP. The D_2O exchange NMR spectra of CPH and DP1was also performed in addition to H^1 NMR for verifying the signals obtained in H^1 NMR. The peak around 4 δ ppm signifies DMSO solvent peak. The absence of singlet due to N-CH_3 and a triplet due to CH_2 of piperidine ring probably signifies breakage of 1-methyl piperidine ring from the CPH structure. (Table 3.12 to Table 3.15 and Figure 3.13 for NMR spectra's of drug and degradation products)

Table 3.12: H^1 NMR Assignments of CPH and DP1

Standard drug			**DP1**		
Position	**Chemical shift (δ ppm)**	**Multiplicity**	**Position**	**Chemical shift (δ ppm)**	**Multiplicity**
1	7.30	d	1	7.44	d
2	7.27	d	2	7.42	d
3	7.25	d	3	7.40	d
4	7.24	d	4	7.20	d
12	7.36	d	12	7.44	d
13	7.28	d	13	7.42	d
14	7.26	d	14	7.40	d
15	7.25	d	15	7.22	d
8	8.27	s	7	7.72	s
22	2.25	s	8	7.75	s
7	7.58	s	16	2.10	d
21	2.73	t	11	7.10	q
20	2.54	t	-	-	-
18	2.57	T	-	-	-
17	2.61	T	-	-	-

Table 3.13: D_20 exchange NMR Assignments of CPH and DP1

Standard drug			**DP1**		
Position	**Chemical shift (δ ppm)**	**Multiplicity**	**Position**	**Chemical shift (δ ppm)**	**Multiplicity**
1	7.25	d	1	7.57	d
2	7.20	d	2	7.41	d
3	7.05	d	3	7.27	d
4	6.81	d	4	7.20	d
12	7.25	d	12	7.59	d
13	7.20	d	13	7.41	d
14	7.07	d	14	7.27	d
15	6.88	d	15	7.21	d
8	7.30	s	7	7.69	s
22	1.95	s	8	7.71	s
7	7.32	s	16	2.05	d
21	3.24, 3.40, 3.42	t	11	7.16	q
20	2.28, 2.31, 2.40	t	-	-	-
18	2.48, 2.52, 2.78	t	-	-	-
17	3.08	t	-	-	-

s-singlet, d-doublet, t- triplet, q- quartet

Table 3.14: C^{13} NMR Assignments of CPH and DP1

Standard drug		**DP1**	
Position	**Chemical shift (δ ppm)**	**Position**	**Chemical shift (δ ppm)**
1	128.18	1	128.75
2	127.99	2	126.47
3	127.85	3	124.28
4	127.66	4	120.23
5	130.67	5	129.03
6	126.70	6	117.83
7	137.33	7	130.14
8	137.33	8	130.74
9	131.07	9	129.03
10	126.70	10	117.83
11	70.02, 70.25, 70.50	11	40.02
12	128.18	12	128.75

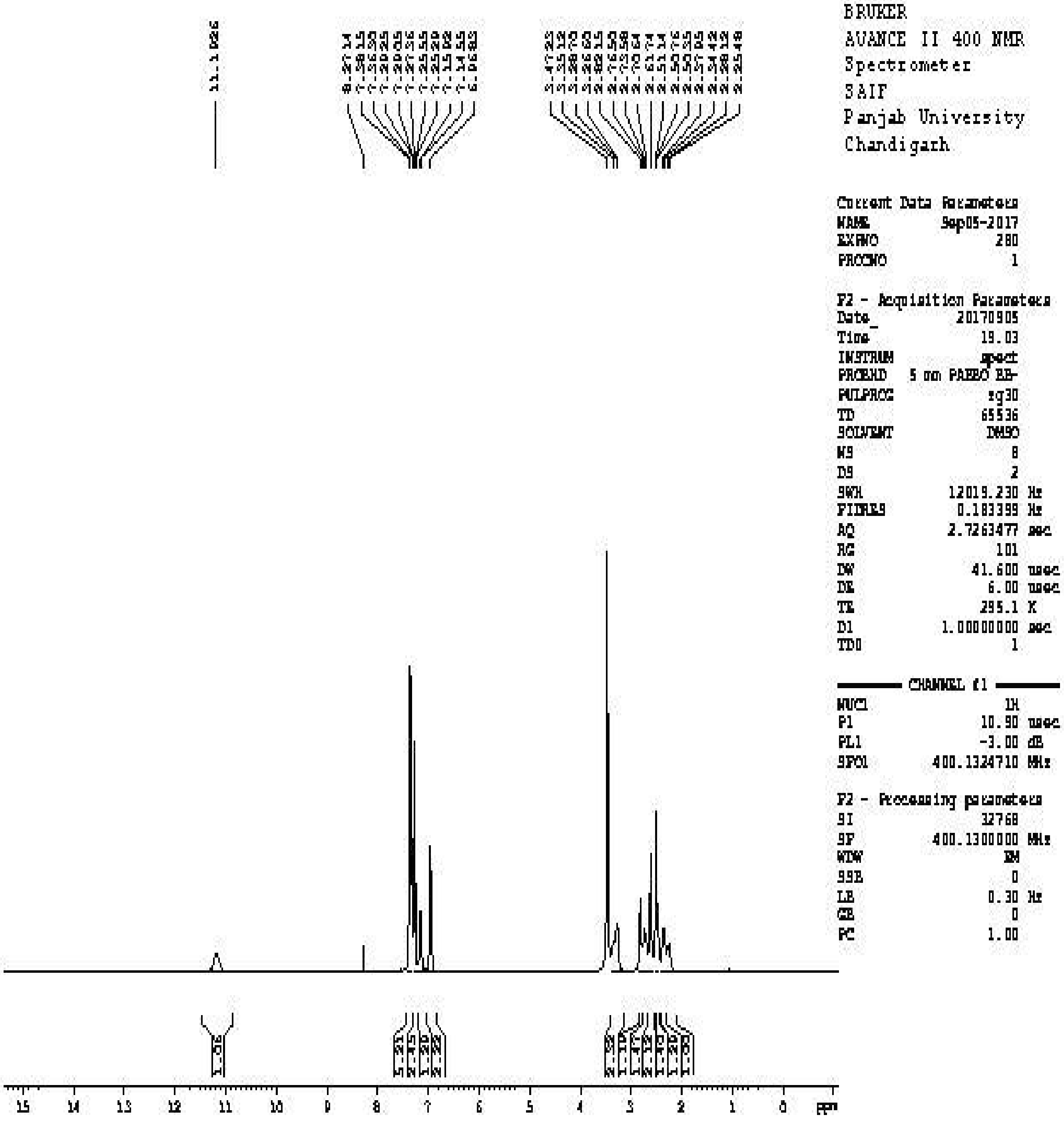
13	127.99	13	126.47
14	127.85	14	124.63
15	127.66	15	120.23
16	55.41	16	16.17
17	40.18	-	-
18	30.02,30.15	-	-
20	30.34,30.55,30.76,30.97	-	-
21	42.12	-	-
22	26.24, 26.55	-	-

Table 3.15: DEPT^{135} NMR Assignments of CPH and DP1

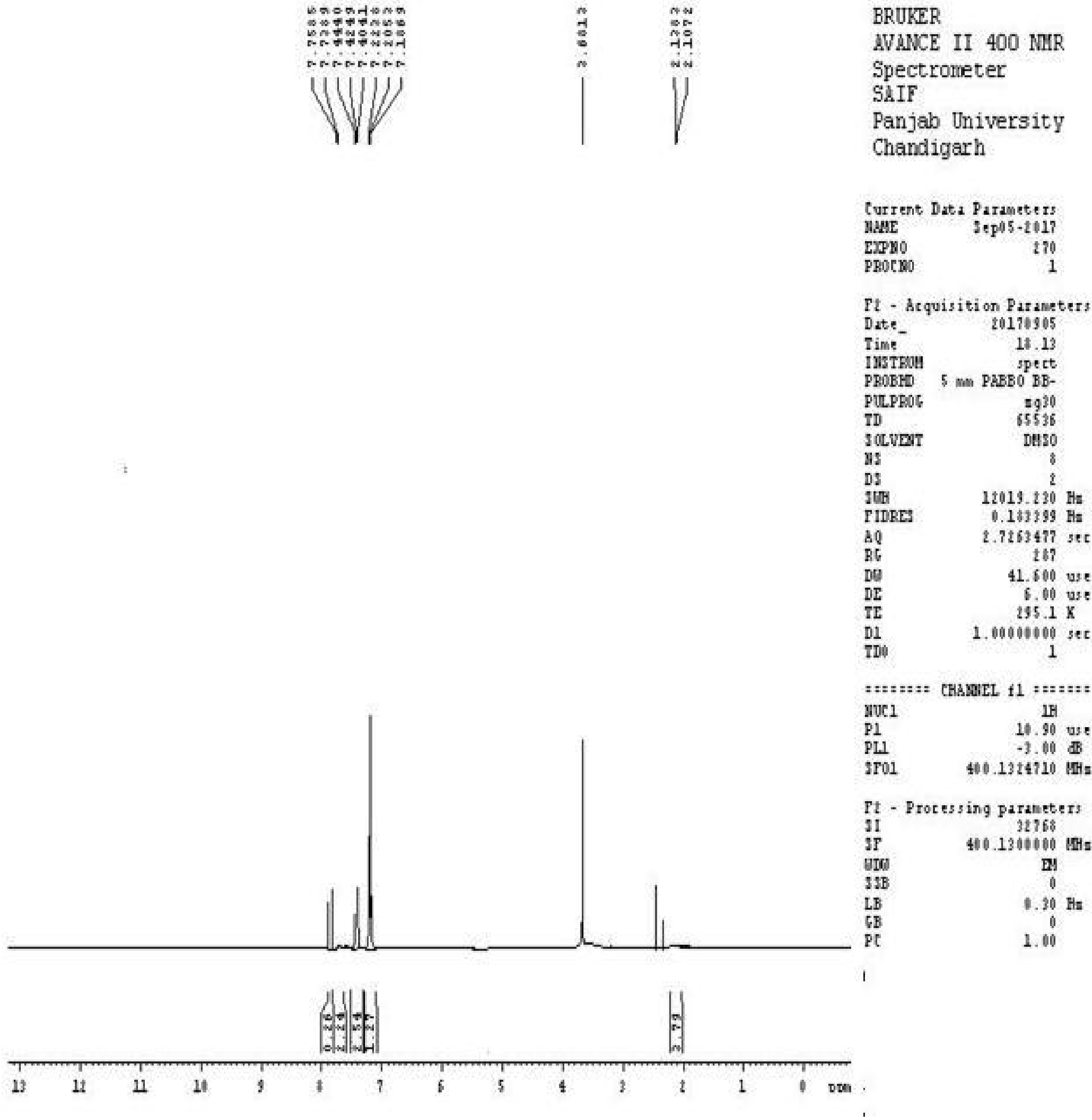
Standard drug			**DP1**		
Position	**Chemical shift (δ ppm)**	**Carbon type**	**Position**	**Chemical shift (δ ppm)**	**Carbon type**
1	128.19 (+)	Tertiary	1	124.45 (+)	Tertiary
2	127.85 (+)	Tertiary	2	124.34 (+)	Tertiary
3	127.66 (+)	Tertiary	3	120.29 (+)	Tertiary
4	126.70 (+)	Tertiary	4	119.41 (+)	Tertiary
7	130.67 (+)	Tertiary	7	128.75 (+)	Tertiary
8	131.07 (+)	Tertiary	8	128.75 (+)	Tertiary
12	128.19 (+)	Tertiary	12	124.45 (+)	Tertiary
13	127.90 (+)	Tertiary	13	124.34 (+)	Tertiary
14	127.66 (+)	Tertiary	14	120.29 (+)	Tertiary
15	126.70 (+)	Tertiary	15	119.41 (+)	Tertiary
17	53.41 (-)	Secondary	16	39.34 (+)	Primary
18	26.24 (-)	Secondary	-	-	
20	26.56 (-)	Secondary	-	-	
21	54.23 (-)	Secondary	-	-	
22	30.50, 30.80 (+)	Primary	-	-	

Figure 3.13 NMR spectra of drug and DP I

a) ^{1}H NMR spectra of CPH



b) ^{1}H NMR spectra of CPH DP1



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BRUKER
AVANCE II 400 NMR
Spectrometer
SAIF
Panjab University
Chandigarh

Current Data Parameters
NAME            Sep05-2017
EXPNO                  270
PROCNO                   1

F2 - Acquisition Parameters
Date_             20170905
Time                 18.13
INSTRUM              spect
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PULPROG               zg30
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SOLVENT               DMSO
NS                       8
DS                       2
SWH              12019.230 Hz
FIDRES            0.183399 Hz
AQ               2.7263477 sec
RG                     287
DW                  41.600 use
DE                    6.00 use
TE                   295.1 K
D1              1.00000000 sec
TD0                      1

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PL1                  -3.00 dB
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F2 - Processing parameters
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WDW                     EM
SSB                      0
LB                    0.30 Hz
GB                       0
PC                    1.00
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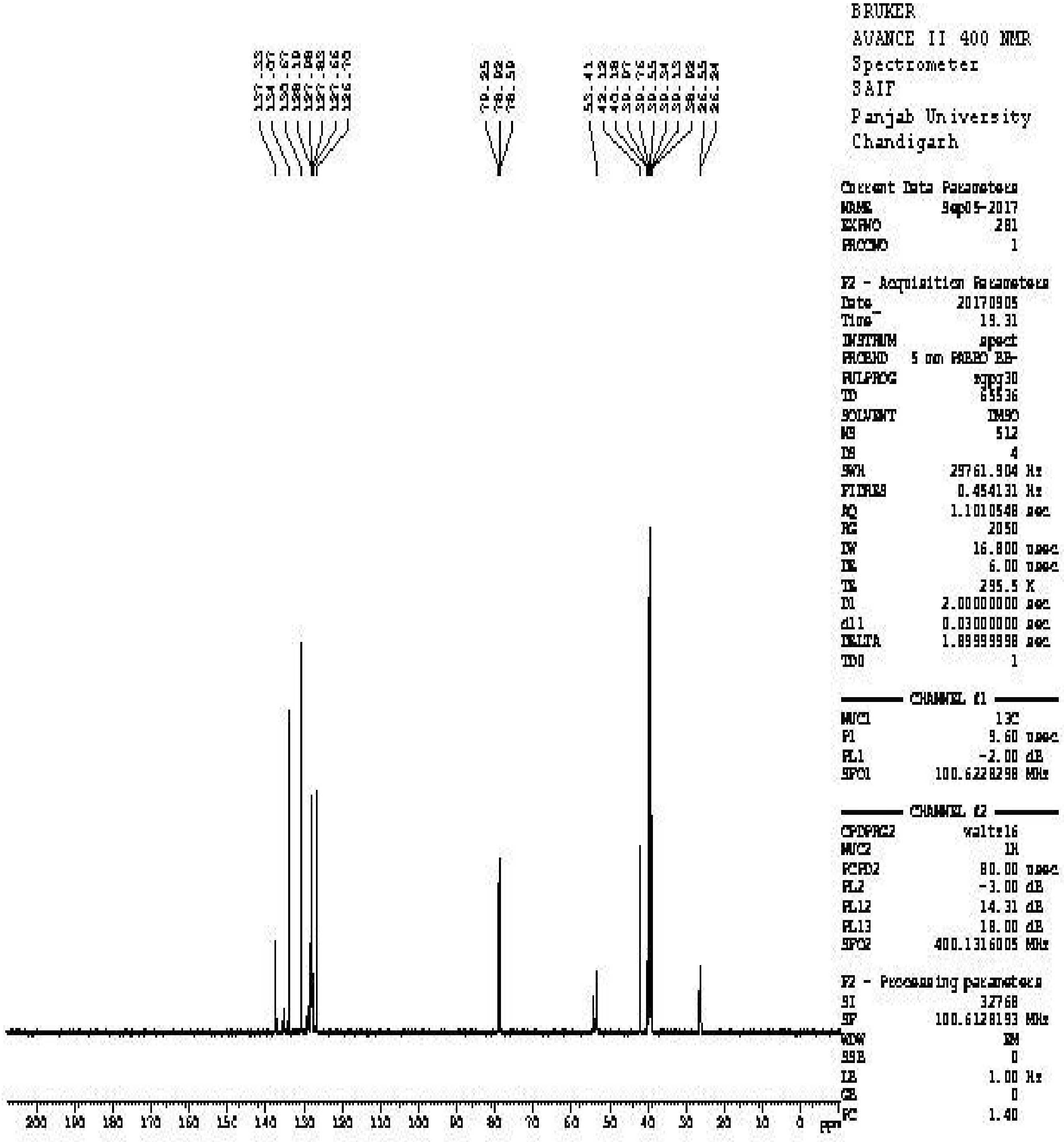
c) ^{13}C NMR spectra of CPH



BRUKER
AVANCE II 400 NMR
Spectrometer
SAIF
Panjab University
Chandigarh

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PROCNO                  1

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SOLVENT             DMSO
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FIDRES          0.454131 Hz
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RG                  2050
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DE                  6.00 usec
TE                 295.5 K
D1            2.00000000 sec
d11           0.03000000 sec
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TD0                    1

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P1                  9.60 usec
PL1                -2.00 dB
SFO1         100.6228298 MHz

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GB                     0
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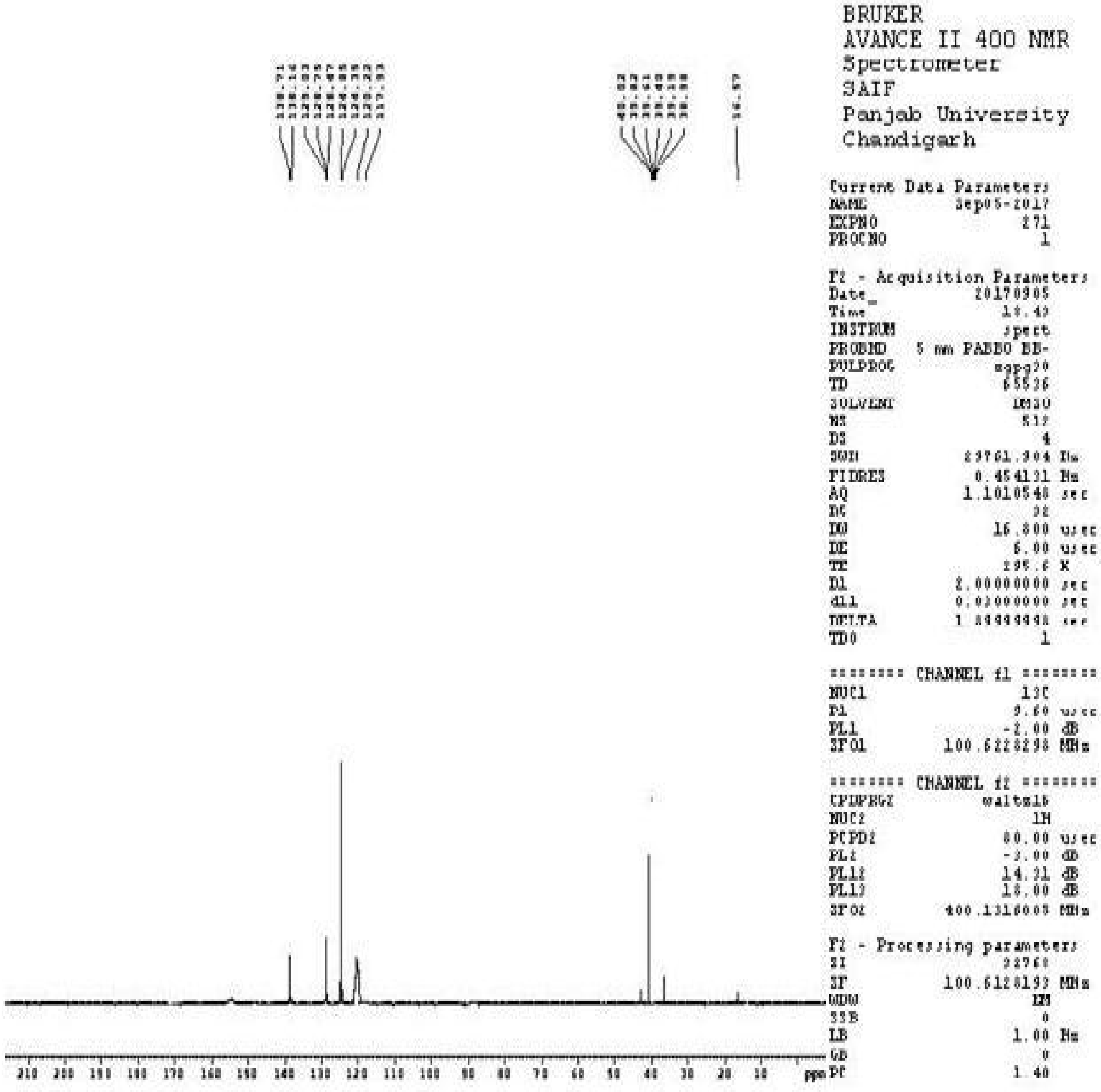
d) ^{13}C NMR spectra of CPH DP1



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BRUKER
AVANCE II 400 NMR
Spectrometer
SAIF
Panjab University
Chandigarh

Current Data Parameters
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SOLVENT             DMSO
NS                   512
DS                     4
SWH            29761.904 Hz
FIDRES          0.454131 Hz
AQ             1.1010548 sec
RG                    32
DW                16.800 usec
DE                  6.00 usec
TE                 295.6 K
D1            2.00000000 sec
d11           0.03000000 sec
DELTA         1.89999998 sec
TD0                    1

======== CHANNEL f1 ========
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P1                  9.60 usec
PL1                -2.00 dB
SFO1         100.6228298 MHz

======== CHANNEL f2 ========
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NUC2                  1H
PCPD2              80.00 usec
PL2                -3.00 dB
PL12               14.31 dB
PL13               18.00 dB
SFO2         400.1316005 MHz

F2 - Processing parameters
SI                 32768
SF           100.6128193 MHz
WDW                   EM
SSB                    0
LB                  1.00 Hz
GB                     0
PC                  1.40
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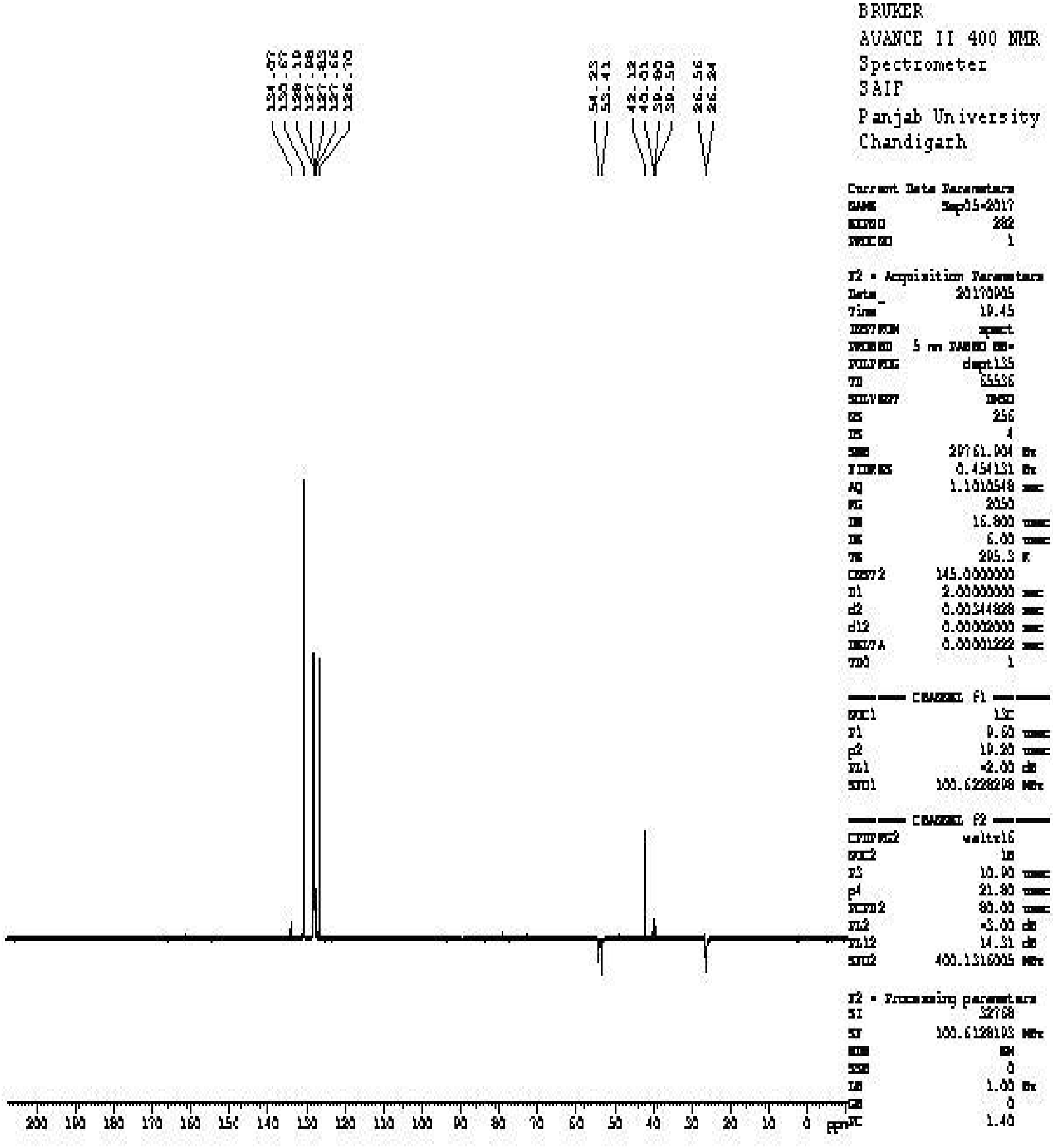
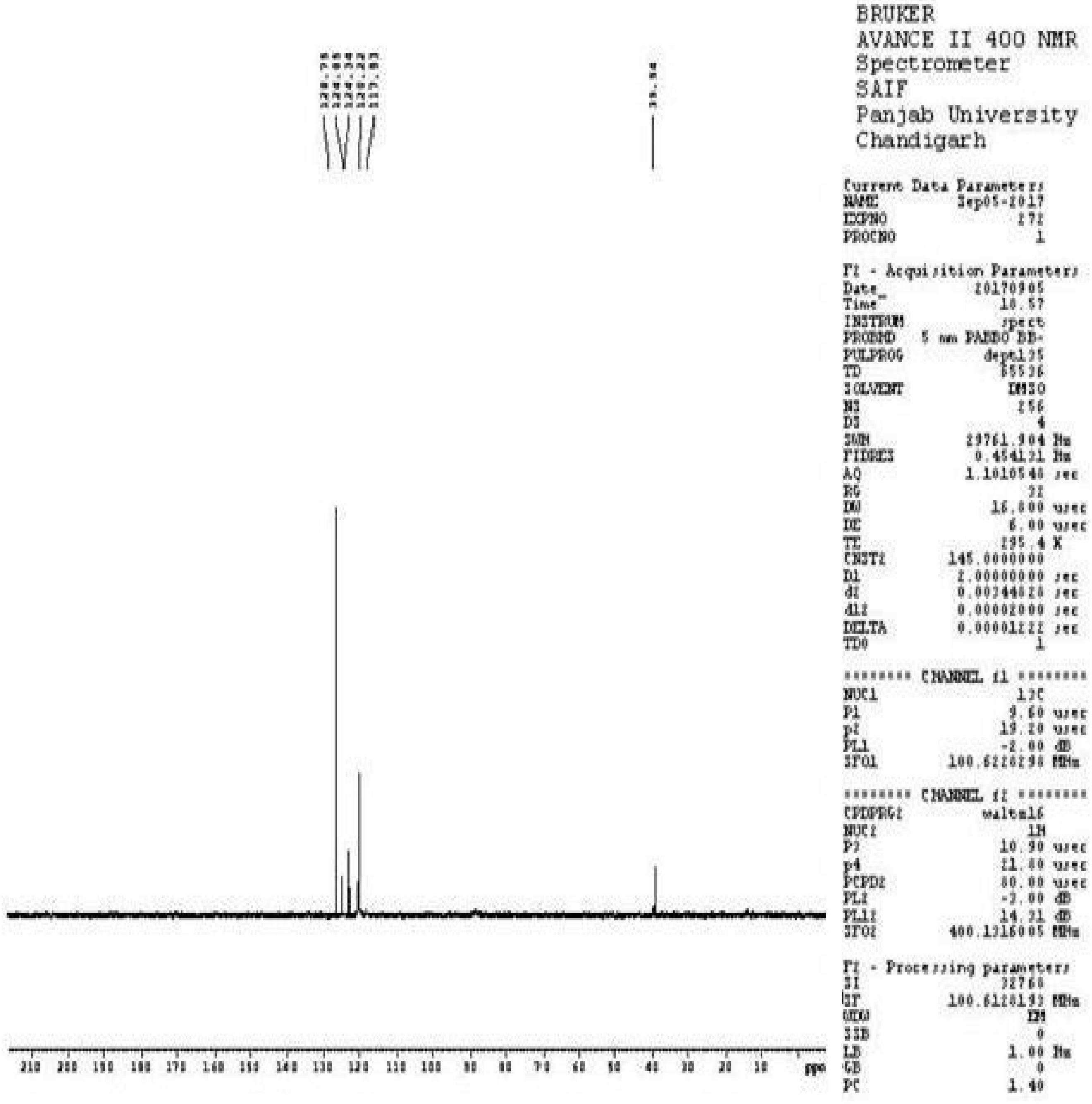
e) ^{135}DEPT NMR spectra of CPH



f) ^{135}DEPT NMR spectra of CPH DP1



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AVANCE II 400 NMR
Spectrometer
SAIF
Panjab University
Chandigarh

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TD                    65536
SOLVENT                DMSO
NS                      256
DS                        4
SWH               29761.904 Hz
FIDRES             0.454131 Hz
AQ                1.1010548 sec
RG                       32
DW                   16.800 usec
DE                     6.00 usec
TE                    295.4 K
CNST2           145.0000000
D1               2.00000000 sec
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d12              0.00002000 sec
DELTA            0.00001222 sec
TD0                       1

======== CHANNEL f1 ========
NUC1                    13C
P1                     9.60 usec
p2                    19.20 usec
PL1                   -2.00 dB
SFO1            100.6228298 MHz

======== CHANNEL f2 ========
CPDPRG2             waltz16
NUC2                     1H
P3                    10.90 usec
p4                    21.80 usec
PCPD2                 80.00 usec
PL2                   -3.00 dB
PL12                  14.31 dB
SFO2            400.1316005 MHz

F2 - Processing parameters
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SSB                       0
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PC                     1.40
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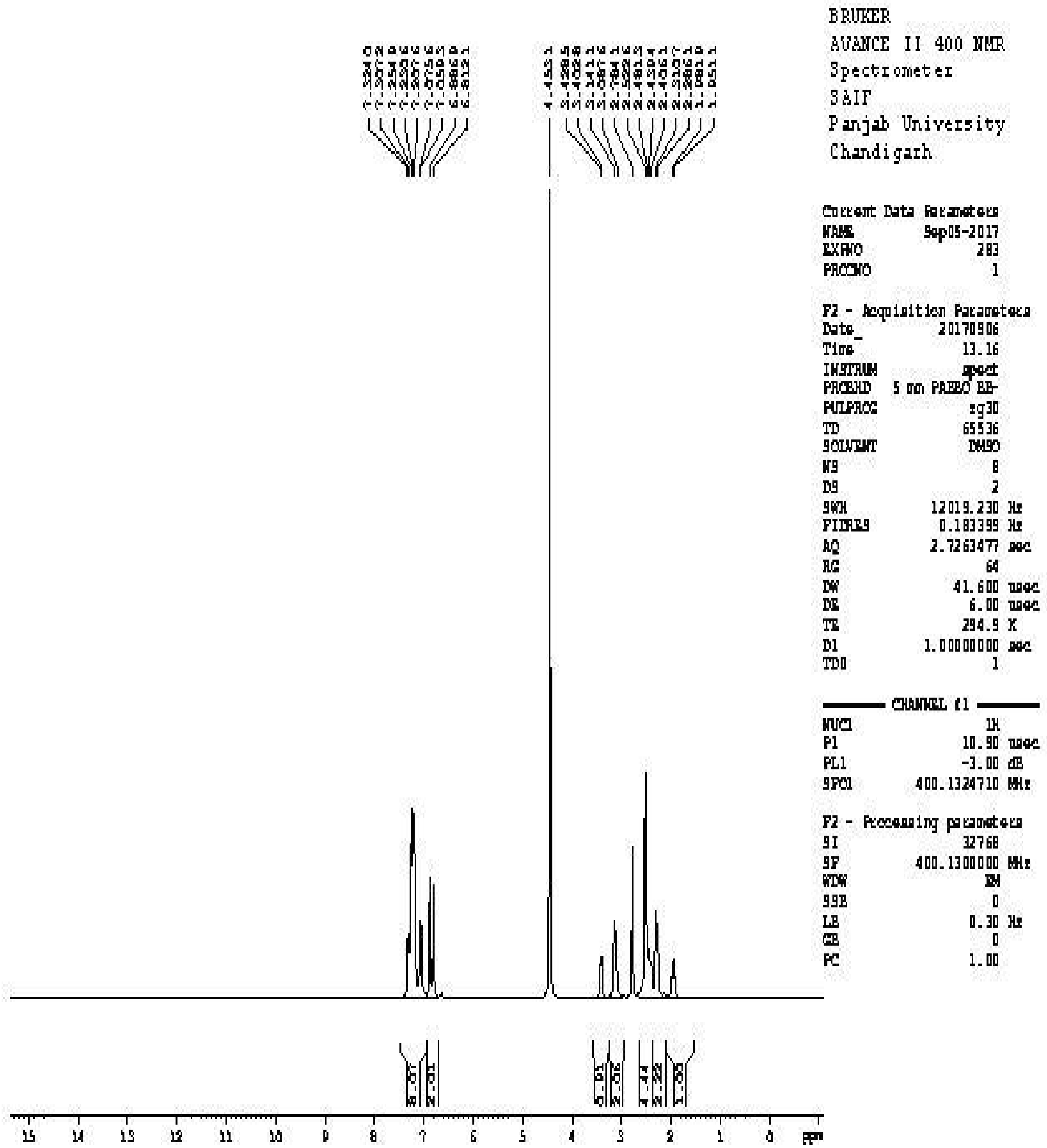
g) D20 exchange NMR spectra of CPH



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Spectrometer
SAIF
Panjab University
Chandigarh

Current Data Parameters
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PROCNO                  1

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FIDRES           0.183399 Hz
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RG                     64
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DE                   6.00 usec
TE                  294.9 K
D1             1.00000000 sec
TD0                     1

======== CHANNEL f1 ========
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PL1                 -3.00 dB
SFO1          400.1324710 MHz

F2 - Processing parameters
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SSB                     0
LB                   0.30 Hz
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PC                   1.00
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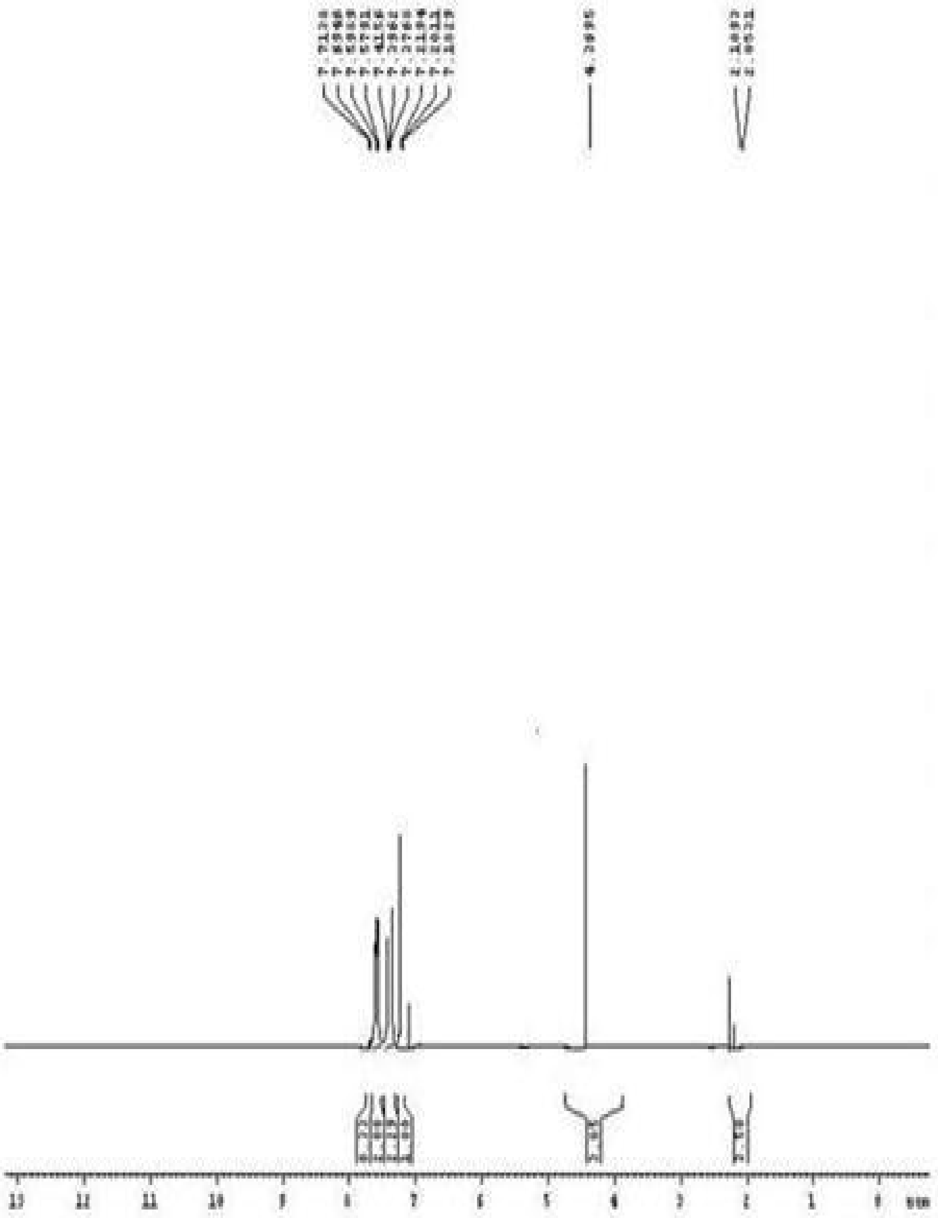
h) D20 exchange NMR spectra of CPH DP1



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BRUKER
AVANCE II 400 NMR
Spectrometer
SAIF
Panjab University
Chandigarh

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PROCNO                 1

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Time               13.31
INSTRUM            spect
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PULPROG          zgpg30
TD                 65536
SOLVENT             DMSO
NS                   512
DS                     4
SWH            23761.904 Hz
FIDRES          0.454131 Hz
AQ             1.1010540 sec
RG                  2050
DW                16.800 usec
DE                  6.00 usec
TE                 295.5 K
D1            2.00000000 sec
d11           0.03000000 sec
DELTA         1.89999998 sec
TD0                    1

======== CHANNEL f1 ========
NUC1                 13C
P1                  9.60 usec
PL1                -2.00 dB
SFO1         100.6228298 MHz

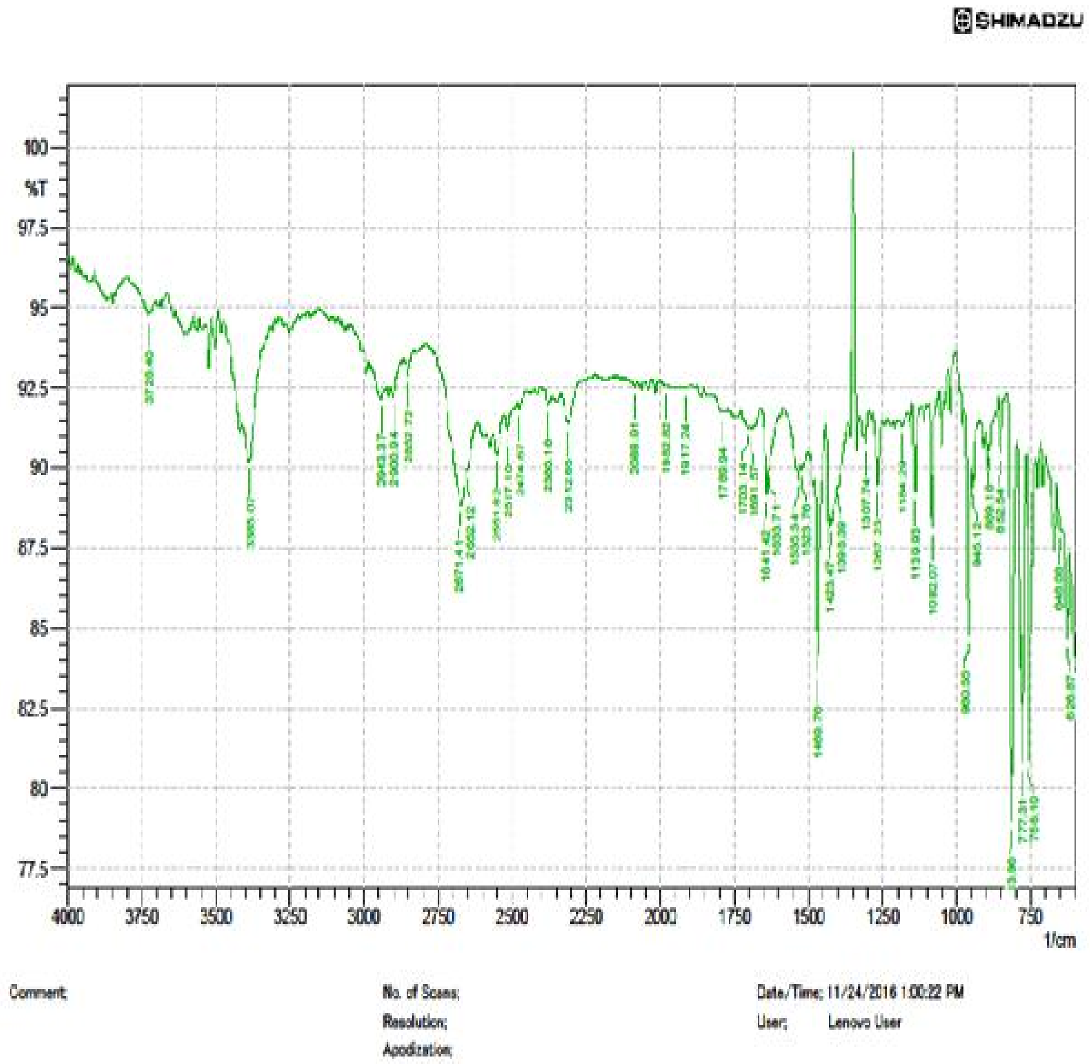
======== CHANNEL f2 ========
CPDPRG2          waltz16
NUC2                  1H
PCPD2              80.00 usec
PL2                -3.00 dB
PL12               14.31 dB
PL13               18.00 dB
SFO2         400.1316005 MHz

F2 - Processing parameters
SI                 32768
SF           100.6128193 MHz
WDW                   EM
SSB                    0
LB                  1.00 Hz
GB                     0
PC                  1.40
```

Further, FT-IR and DSC studies were also done for more comprehensive characterization of structure elucidation. The FT-IR analysis of CPH and the isolated DP1 was carried out and the FTIR spectra showed marked difference in the frequencies observed for DP1 when compared with the CPH frequencies as shown in Figure. 3.14. The absence of frequency 3385 cm^{-1} (corresponding to NH streching) and of frequency 1614 cm^{-1} (corresponding to NH bending) in DP1 inferred the absence of 1-methyl piperidine ring in the CPH drug structure. (Table 3.16)

Figure 3.14 IR spectra

a) IR spectra of CPH showing its characteristics frequencies



b) IR spectra of CPH DP1 showing its characteristics frequencies

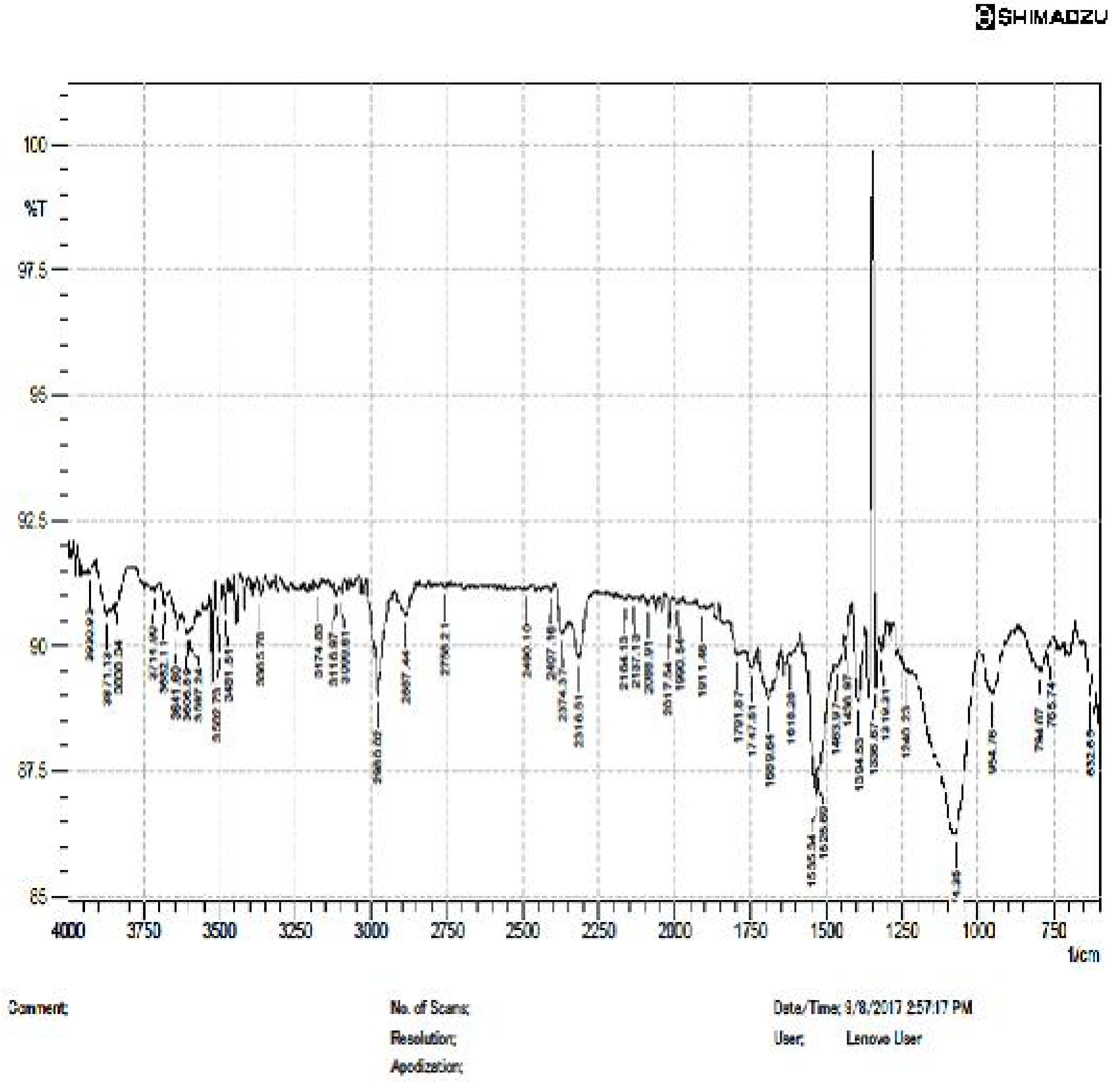


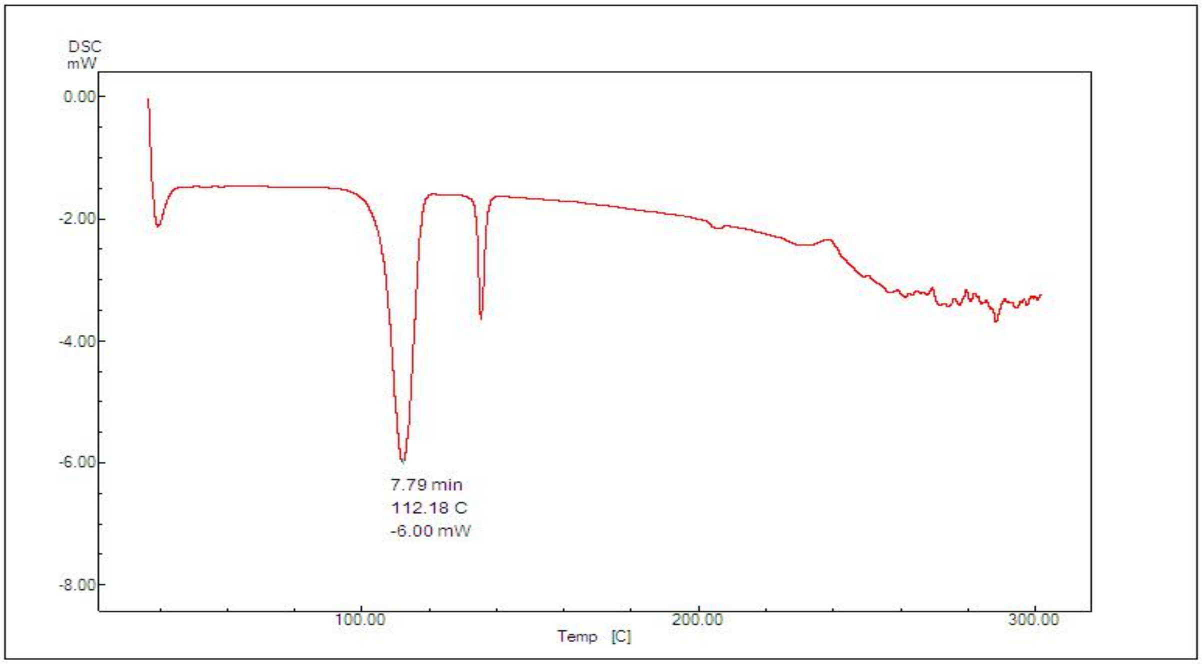
Table 3.16 FTIR Spectral Data of CPH and DP1 (Refer Figure 3.19 for IR spectra)

CPH		DP1	
Wave number(cm^{-1})	Functional group	Wave number(cm^{-1})	Functional group
3385	NH stretching	2990	CH stretching
2671	CH stretching	2316	C=C stretching
1614	NH bending	1535	Aromatic ring
1469	Aromatic ring	1004	C-C stretching

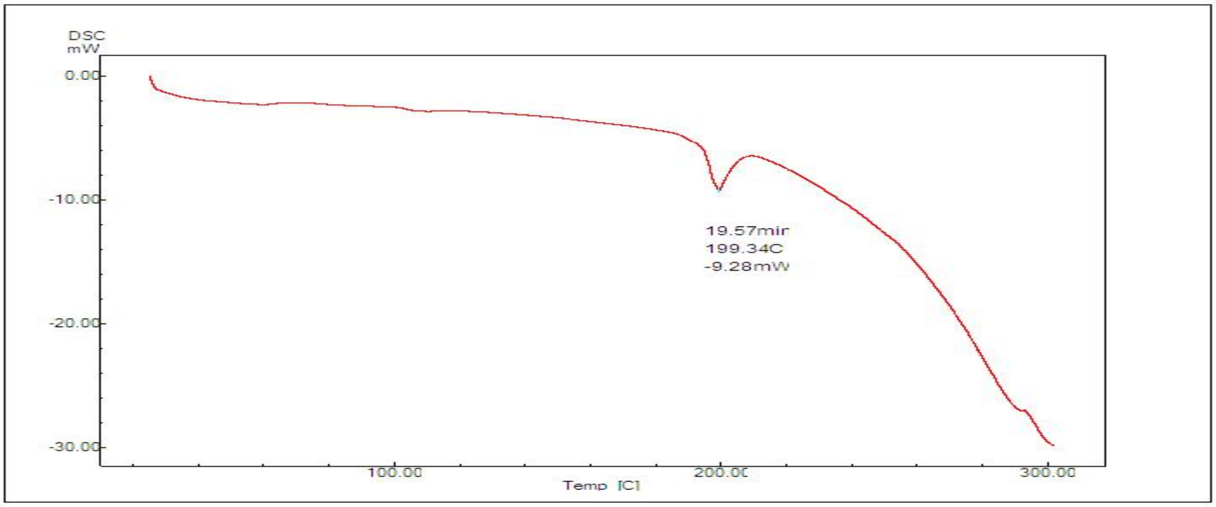
DSC thermogram of both CPH and DP1 was taken. The DSC thermogram of CPH showed an endotheric peak at 112.18 °C which indicates that it melts at this temperature. Similarly in DP1, one endothermic peak was observed at 199.34°C indicating its melting temperature. The change in endothermic peak of DP1 from CPH signifies a structural change of DP1 from standard drug CPH. (Figure. 3.15 for DSC thermogram of drug and DP I)

Figure 3.15 DSC thermogram of CPH and CPH DP I showing its endothermic peak

a) DSC thermogram of CPH



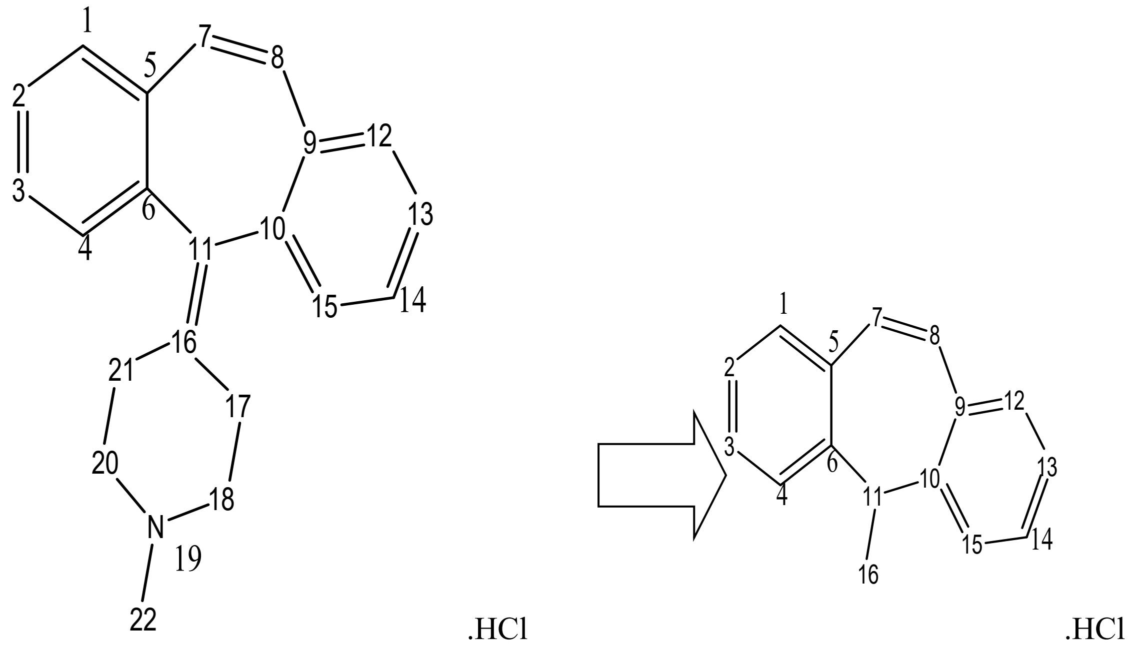
b) DSC thermogram of CPH DP1 showing its endothermic peak



3.5.2.2 Proposed degradation pathway [21]

The piperidine ring of parent molecule gets hydrolyzed and gets cleaved to give a moiety with m/z of 239 gm/mol. The proposed IUPAC name of the unknown DP1 is 4-(dibenzo [1, 2-a: 1’, 2’-e] [7] annulen-11-ylidene). (Figure. 3.16)

Figure 3.16 Proposed degradation pathways



Drug **DP1 and DP2**

3.6 CONCLUSION

Degradation study of CPH was carried out systematically with LC-PDA detection and LC-MS/MS analysis. One each degradation related impurities were observed under acidic and basic condition in LC-PDA but as per LC/MS/MS analysis the two impurities indicated these to be similar in structure as per m/z values. This one major degradation product formed from hydrolysis degradation condition was isolated and successfully characterized by FT-IR, Mass and NMR spectral studies. The chemical name of isolated degradation product at retention time of 2.8 min is 4-(dibenzo [1, 2-a: 1', 2'-e] [7] annulen-11-ylidene) which is hitherto unreported in literature. The structure and degradation pathway of this degradation product was proposed on the basis of LC-MS/MS, FT-IR and NMR analysis. A stability indicating RP-HPLC method for identification and quantification of CPH and DP1 was developed and validated as per ICH Q2 (R1) guideline. The method is simple, sensitive, and accurate and fast which is applicable for the assay of CPH and estimation of DP1.

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