DEVELOPMENT OF STABILITY INDICATING METHOD OF ACOTIAMIDE HCL TRIHYDRATE

4.1. SELECTION OF DRUG

Acotiamide (ACOT) is the first drug in the category of prokinetic agent used in the treatment on functional dyspepsia. It was developed by Zeria Pharmaceuticals Co. Ltd. and Astellas Pharma Ltd and it was first approved by Japan in 2013 [1]. It was approved by CDSCO in India in 2016. It is not official in any pharmacopoeia. It acts an antagonist on M1 and M2 muscarinic receptors and cause inhibition of acetylcholinestrase activity and there is increase in release of acetyl choline. ACOT is well tolerated and has benefits of meal related symptoms like postprandial fullness, early satiety [2, 3]. It has low affinity to dopamine D2 or serotonin [4]. Literatures have reported that in comparison to itopride and mosapride, ACOT increases gastric antral motility, and improve hypomotility and delayed gastric emptying without affecting QT interval [5, 6]. ACOT produces alteration in stress related genes like GABA receptors, GABA transporters and neuromedlin U in hypothalamus. It has importance role in regulation of stress through hypothalamic-pituitary-adrenocortical axis activity [7, 8]. There are clinical trials which have shown that ACOT in combination with rabeprazole relieved symptoms in patients with GERD and FD with heartburn and epigastric fullness [9]. It is safe and effective drug in comparison to levosulpiride [10]. Daily dose of ACOT tablet is 100 mg three times a day [11, 12]. No SIAM could be found during thorough literature survey done before undertaking the study but during the conduct of research work, a research paper was published reporting the degradation data of ACOT by UPLC-Q-TOF [21]. In present study, it was tried to develop a stability indicating method based on HPLC. Efforts were also made for identification of degradation products by LC-MS and isolation of two degradation products and characterization by mass, NMR and IR techniques.

4.2. DRUG PROFILE

General Properties

IUPAC name: N-[2-[di (propan-2-yl) amino] ethyl]-2-[(2-hydroxy-4, 5-dimethoxybenzoyl) amino]-1, 3-thiazole-4-carboxamide; trihydrate; hydrochloride [13]

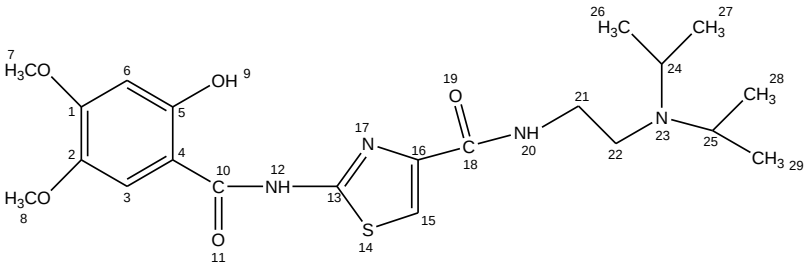


Fig. 4. 1 – Structure of ACOT

Molecular Formula: $C_{21}H_{30}ON_4O_5S.HCl.3H_2O$

Molecular Weight: 541.06 g

Appearance: white amorphous powder

Melting Point: 144ºc

Log P: 2.06

pKa: 10.24

Solubility: water, methanol and acetonitrile

Drug Category: Prokinetic agent

Mechanism of action:

ACOT produces its action by inhibition of acetylcholinestrase activity in the stomach, as a result there is increase in release of acetylcholine. It causes reversible inhibition of acetylcholinestrase and inhibition of presynaptic M1 and M2 receptors, there is a release of acetylcholine from the nerve terminal of enteric nervous system. Increase in gastric accommodation is observed after meal digestion with ACOT. It acts on the afferent vagus nerve and produces changes in the brain-gut interactions, changes in the sensory input from GI tract to the CNS or vasovagal reflex pathways [14].

Uses: in the treatment of functional dyspepsia [15]

Marketed Formulation: Acogut tablet 100mg (Lupin)

4.3. LITERATURE REVIEW

The literature already reported is summarized here:

- *Determination of industrial acotiamide hydrochloride trihydrate by high performance liquid chromatography by Zhou Wei et.al. [16]*

The method is developed on C 18 column (150 mm x 4.6 mm, 5 µm) with 20 mM ammonium acetate pH 6.8 and methanol in the ratio of 55: 45. Detection was performed at 280 nm. Retention time of ACOT is 8 min and total analysis time is 23 min. The method can be performed for rapid detection of ACOT and used in the quality control of raw material.

- *RP- HPLC method was developed for the determination of acotiamide hydrochloride in bulk and tablet by Vani R et. al. [17]*

Method was developed for ACOT by RP-HPLC method. Chromatographic separation was achieved on Agilent C 18 column (150mm x 4.6 mm, 5 µm). Mobile phase was methanol and acetonitrile in the ratio of 60: 40. Detection was performed at 235 nm. Flow rate was maintained at 1 mL/min. Retention time was found to be 2.825 min. The method was validated as per ICH guidelines. The method can be used for routine analysis of ACOT in API and pharmaceutical dosage form.

- *LC-MS/MS method was developed for the determination of acotiamide in rat plasma by Li J et. al. [18]*

LC-MS/MS method has been developed for the analysis of ACOT in rat plasma. Precipitation was performed with methanol-acetonitrile (50:50) and gradient elution with mobile phase containing water containing 0.1% formic acid and methanol. Chromatographic separation was achieved on Agilent Zorbax XDB C 18 column (50 mm x 2.1 mm, 3.5 µm). Flow rate was maintained at 0.5 mL/min. The analytes were monitored by tandem-mass spectrometry with positive electrospray ionization. The developed method was applicable for pharmacokinetic evaluation of ACOT after intravenous and oral administration of 10 mg/kg ACOT in rats.

- *LC-MS/MS method was developed for the determination of acotiamide in rat plasma by UHPLC-Q-TOF-MS. The developed method was validated and applied for pharmacokinetics study by Patel P et.al. [19]*

UPLC –ESI mass spectrometry method was developed and validated for the quantification of ACOT in rat plasma. For extraction of ACOT from rat plasma,

acetonitrile was used for precipitation method. ACOT and internal standard Mirabegron were separated on Agilent poroshell EC C 18 column (50 mm x 3.0 mm, 2.7 µm) using 10 mM ammonium acetate : methanol in binary gradient at a flow rate of 0.4 mL/min over 4 min run time. Detection was performed for ACOT and internal standard in selective ion mode. UPLC-MS method can be extended to measure plasma concentrations of ACOT in human to understand drug metabolism, drug interaction and adverse effects.

- *Identification of in vivo metabolites of acotiamide in rats using ultra performance liquid chromatography –quadruple/time of flight mass spectrometry by Patel P et.al. [20]*

The metabolism of ACOT was investigated using liquid chromatography positive ion electrospray ionization high resolution mass spectrometry. The structural elucidation of the metabolites was performed by comparing their $[M+H]^+$ ions and their product ions with those of parent drug. Total seven hitherto unknown metabolites were characterized from the biosamples.

- *Development of stability indicating assay for acotiamide by ultra –high-performance liquid chromatography /electro spray ionization quadruple time of flight tandem mass spectrometry, identification and characterization of degradation products and its process related impurity by Thummar M et. al. [21]*

ACOT was subjected to stress degradation under hydrolytic, oxidative, photo and thermal stress conditions. Degradation products as well as a process related impurity were separated from the drug on Waters Acquity HSS cyano column (100 x 2.1 mm, 1.8 µm). Gradient elution was performed for separation of degradation products using 0.1% formic acid and acetonitrile .Flow rate was maintained at 0.25 mL/min.

The drug degraded under hydrolytic (acidic and basic), oxidative and photolytic stress while it was stable under neutral hydrolytic and thermal stress conditions. Seven degradation products and one process related impurity were observed. The method was validated as per ICH guidelines.

4.4. SECTION - A

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPLC METHOD

4.4.1. EXPERIMENTAL

4.4.1.1. Chemicals and Reagents

- ACOT bulk drug was provided as gift sample from Hetero Drugs Pvt. Ltd, Hyderabad.
- Commercial tablet dosage form Acogut 100 mg were purchased from local pharmacy
- Acetonitrile (ACN) of HPLC grade was purchased from Rankem Pvt. Ltd. Mumbai.
- HPLC grade formic acid, triethylamine were procured from Loba Chemie Pvt. Ltd. Mumbai.
- Hydrochloric acid (HCl), sodium hydroxide (NaOH), hydrogen peroxide H_2O_2) were purchased from S.D. Fine Chemical Ltd. Mumbai.
- 0.22 µm Nylon 6,6 membrane filter , Ultipore® N,66® for filtration of mobile phase was procured from Pall Life Sciences ,USA.
- 0.45 µm Nylon 6,6 syringe filter for sample filtration was procured from Pall life Sciences , USA.

4.4.1.2. Equipments and Instruments

Apart from equipments and instruments mentioned in section 3.4.1.2. Other equipments and instruments used are:

- **High Performance Liquid Chromatography (HPLC)**: HPLC system comprised of Waters Alliance HPLC system equipped with 2695 separation module, Photo Diode Array Detector (PDA) (model 2996). Emchem 2 software was used for data acquisition and integration.
- **Mass spectroscopy:** LC/MS/MS studies were performed with LCQ fleet, Thermo Fischer scientific instrument having quaternary system delivery module with positive and negative ESI (electro spray ionization) mode. The electro spray parameters were gas temperature at 250ºC, nitrogen as drying

gas at 30 psi and capillary voltage of 5500V, nebulizer pressure using nitrogen gas at 20 psi. Data acquisition was done with Xcalibur software.

- **IR spectroscopy :** IR spectra was recorded on Shimadzu ATR-FTIR spectrophotometer
- **NMR spectroscopy:** NMR (^{1}H and ^{13}C) experiments of ACOT and its degradation products were performed on Bruker 400 MHz NMR spectrometers using DMSO-d_6 and $CDCl_3$ solvents. Tetramethylsilane was used as internal standard. For ^{1}H NMR chemical shift values were recorded on the δ scale in ppm, with respect to TMS (δ=0.00 ppm) and for ^{13}C chemical shift values were recorded with respect to DMSO-d_6 (δ= 39.50 ppm) and $CDCl_3$ (δ= 77.0 ppm). Distortionless enhancement by polarization transfer (DEPT) spectral indicated presence of methylene as negative peaks, methyl and methine groups as positive peaks. The data was recorded with Linux software.

4.4.1.3. Chromatographic conditions

Buffer used in mobile phase was 0.1% triethylamine in 0.2% formic acid, prepared by dissolving 1mL of triethyl amine in 1000 mL of double distilled water. Formic acid was used to adjust pH of buffer (3.0). Mobile phase was prepared by mixing buffer and acetonitrile (70: 30). Before Mobile phase was filtered with 0.2µ membrane filter, prior to use and for degassing it was sonicated for 5 min. Analysis was performed with column oven temperature at 40ºC with detection wavelength of 282 nm and flow rate of 1mL/min. The injection volume was 20µL. Analysis was performed on Thermo Hypersil BDS C-8 column (250 mm x 4.6 mm i.d. x 5µm particle size).

4.4.1.4. Preparation of Standard solution

ACOT standard solution (1mg/mL) - 25 mg of ACOT was weighed accurately and transferred to 25 mL volumetric flask, dissolved in water and acetonitrile (70 : 30) and volume was made up to the mark.

ACOT working standard solution was prepared at concentration of 100µg/mL from stock solution in mobile phase.

Calibration curves were prepared in the concentration ranging from 5 to 30µg/mL from working standard solution.

4.4.1.5. Preparation of forced degradation sample

For forced degradation study, stock solution of ACOT (1mg/mL) was prepared in water.

4.4.1.5.1. Acid degradation

5 mL of ACOT stock solution was transferred to 25 mL of volumetric flask, to this was added 1 mL of 1 M HCl. The solution was heated at 100ºC for 3 hrs. The solution was neutralized with 1mL of 1 M NaOH and volume was made up to 25 mL with water to make the concentration of 200µg/mL. The solution was filtered through 0.45 µ Nylon 6, 6 syringe filter before injecting into HPLC system.

4.4.1.5.2. Alkaline degradation

5 mL of ACOT stock solution was transferred to 25 mL of volumetric flask, to this was added 1 mL of 0.5 M NaOH. The solution was heated at 100ºC for 3 hrs. The solution was neutralized with 0.5 M HCl and volume was made up to 25 mL with water to make the concentration of 200µg/mL. The solution was filtered through 0.45 µ Nylon 6, 6 syringe filter before injecting into HPLC system.

4.4.1.5.3. Oxidative degradation

5 mL of ACOT stock solution was transferred to 25 mL of volumetric flask, to this was added 1 mL of 6% hydrogen peroxide. The solution was kept at room temperature for 48 hrs. The volume was made up to 25 mL with water to make the concentration of 200µg/mL. The solution was filtered through 0.45 µ Nylon 6, 6 syringe filter before injecting into HPLC system.

4.4.1.5.4. Neutral hydrolysis degradation

5 mL of ACOT stock solution was transferred to 25 mL of volumetric flask, to this was added 1 mL of water. The solution was heated at 100ºC for 6 hrs. The volume was made up to 25 mL with water to make the concentration of 200µg/mL before injecting into HPLC system.

4.4.1.5.5. Dry heat degradation

For dry heat degradation, 25 mg of ACOT was kept in oven at 80ºC for 11 days. The sample was transferred to 25 mL of volumetric flask, dissolved in water. From this, concentration of 200 µg/mL of solution was prepared and injected into HPLC system.

4.4.1.5.6. Photolytic degradation (Dry)

For photolytic degradation, 25 mg of ACOT was spread in 1 mm thickness and was exposed in photolytic chamber for 11 days. Volume was made up to 25 mL and from this, concentration of 200µg/mL was prepared and injected into HPLC system.

4.4.1.5.7. Photolytic degradation (Solution)

ACOT (1mg/mL) solution was exposed in photolytic chamber for 11 days. From this, concentration of 200µg/mL was prepared and injected into HPLC system.

4.4.1.6. HPLC method validation

The developed method was validated as per ICH Q2B guideline.

For linearity, standard dilutions of ACOT were prepared in the concentration ranging from 5 to 30µg/mL from ACOT stock solution and were injected in triplicate. Linearity was determined by plotting peak area and concentration of solution. From the graph regression equation and regression coefficient was determined.

For precision, intra-day and inter-day precision were evaluated at six concentration levels (in triplicates). Peak areas corresponding to the concentration was calculated and % RSD was determined for intra-day and inter -day precision.

% Recovery was evaluated by standard addition method. Accuracy of method was confirmed by recovery study from formulation at 3 level of standard addition (50%, 100% and 150%). Accuracy of method was evaluated at concentration of 20µg/mL. Final concentrations for accuracy were 20, 30, 40, 50µg/mL. The concentrations were analysed in triplicates. % recovery and % RSD were calculated.

Limit of detection and limit of quantitation were calculated on the basis of standard deviation of the intercept and slope of the calibration curve. LOD and LOQ were calculated using equation 3.3*(σ/S) and 10*(σ/S), where σ is the standard deviation of intercept and S is the slope of the calibration curve.

For robustness, factors like percentage of acetonitrile in the mobile phase (28, 30, 32), flow rate (0.9, 1.0, 1.1mL/min), wavelength (281,282,283 nm) and column oven temperature (38, 40, 42 ºC) were changed. Robustness of the method was evaluated at 15 µg/mL of concentration in triplicates.

The standard stock solution of ACOT was kept for 24 hours at room temperature. The stability of stock solution was determined.

Specificity of the method was evaluated by analysis of API as well as in formulation along with degradation products and to check the method for interference of any peaks affecting the estimation of ACOT .

For system suitability tests six replicate solutions were injected. The parameters retention time, asymmetry factor and theoretical plates were noted.

4.4.2. RESULTS

4.4.2.1. Determination of suitable wavelength

ACOT solution of 10µg/mL was prepared and was scanned in the UV region of 200-400 nm ACOT showed strong absorbance at 282nm which was selected as the analytical wavelength (Fig. 4.2).

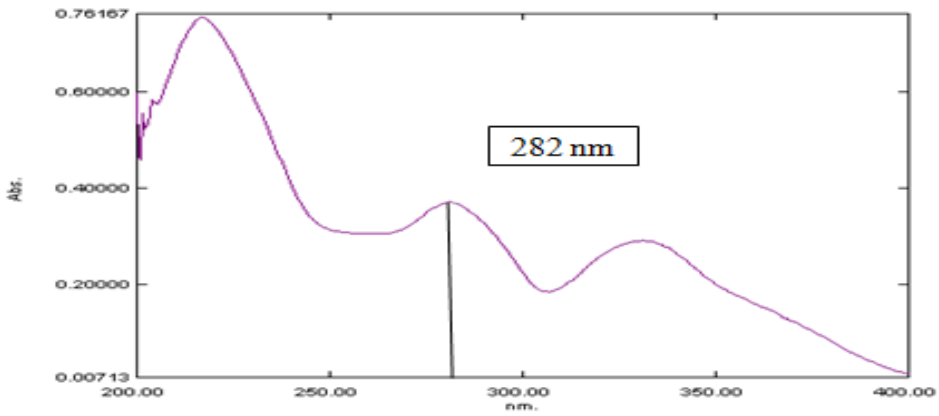


Fig. 4. 2 – Zero order spectra of ACOT (10µg/mL)

4.4.2.2. Method optimisation and development

For optimisation of chromatographic conditions, effect of chromatographic parameters like pH of mobile phase, composition of mobile phase and flow rate were studied. The chromatograms were recorded and chromatographic parameters like theoretical plates, asymmetric factors were recorded. The results of the trials are shown in Table 4.1.

Table 4. 1– Optimisation of HPLC conditions

Mobile phase	Ratio	Column	Flow rate	Retention time	Peak Shape
Water : Methanol	50 : 50	Phenomenex C-18 column	1 mL/min	No peak up to 30 min	
Phosphate buffer : Methanol (pH 6)	50 : 50	Phenomenex C-18 column	1 mL/min	16.053 min	Broad Peak
Phosphate buffer : Methanol (pH 4)	50 : 50	Phenomenex C-18 column	1 mL/min	18.480 min	Broad Peak
Formate buffer : Methanol (pH 4.0)	40: 60	Phenomenex C-18 column	1 mL/min	4.402 min	Peak bifurcation
Formate buffer : Methanol(pH 4.0)	50 : 50	Hypersil BDS C-18 column	1 mL/min	9.276 min	Slight tailing
0.1% Formic acid Methanol	50 : 50	Hypersil BDS C-18 column	1 mL/min	8.012	Sharp Peak

Initially method was optimised with 0.1% formic acid and methanol in the ratio of 50:50. During development of stability indicating method, one of the degradation products in alkaline condition was co-eluting with ACOT (Fig.4.3). To resolve these degradation products, trials were taken with changing mobile phase and other column. Finally degradation product was resolved from ACOT on C-8 column and with mobile phase 0.1% TEA in 0.2% formic acid and acetonitrile in the ratio of 70: 30 (Fig. 4.4). The optimised chromatogram of ACOT is shown in Fig. 4.5. ACOT eluted at retention time of 9.36 min (Table 4.2).

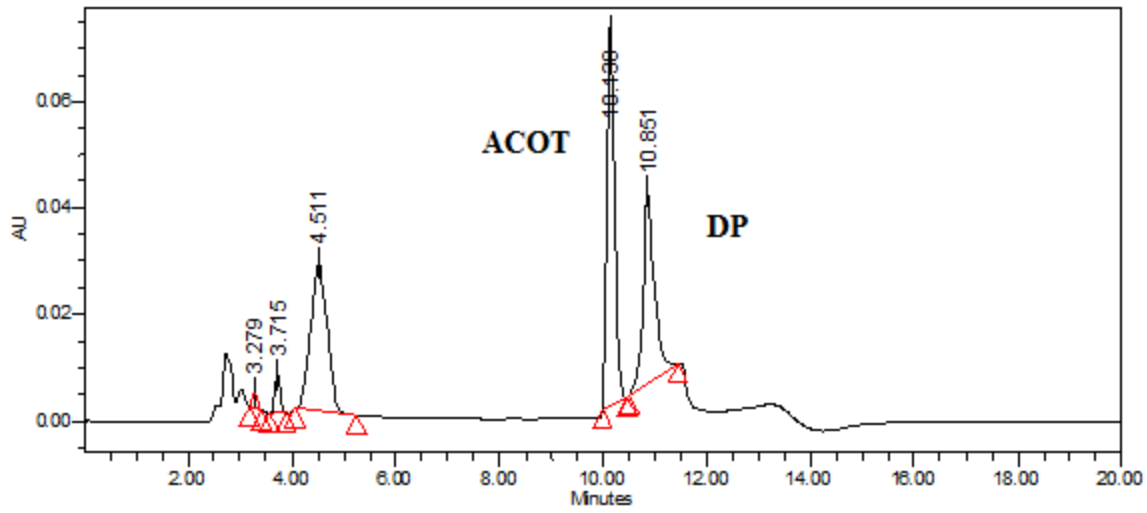


Fig. 4. 3- Chromatogram of 0.5 M NaOH condition (co-elution of DP and ACOT, C-18 column)

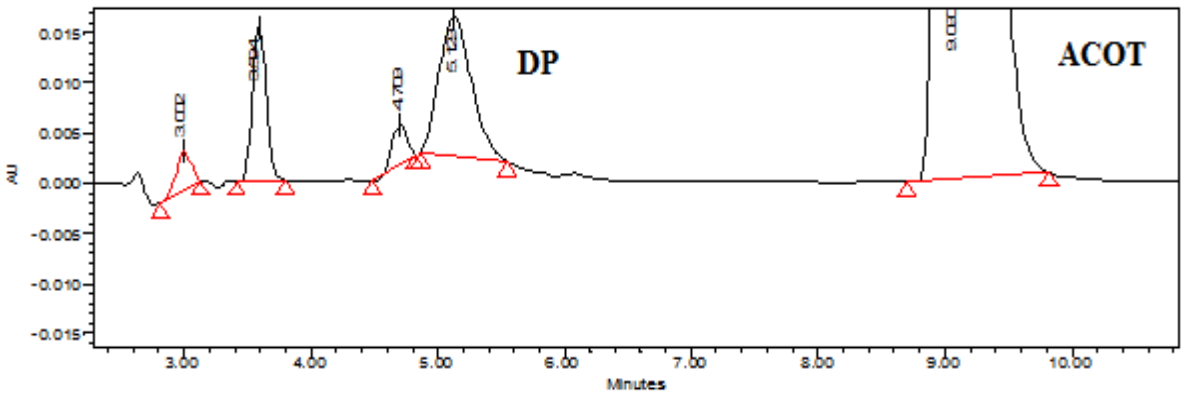


Fig. 4. 4 – Chromatogram of 0.5 M NaOH condition on C-8 column

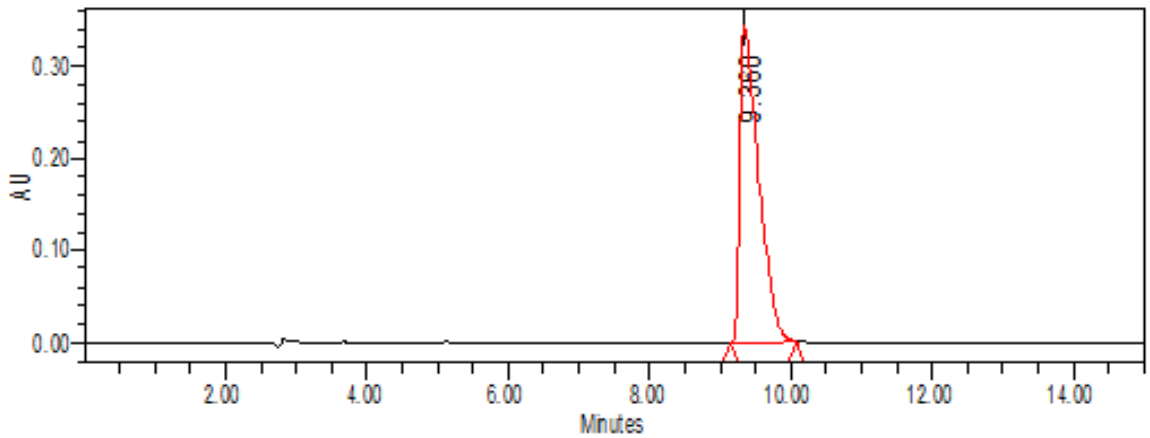


Fig. 4. 5- Chromatogram of ACOT (200µg/mL)

Table 4. 2 – Optimised HPLC parameters

Parameters	Optimised Value
Column	Thermo Hypersil BDS C-8 (250 mm x 4.6mm i.d. , 5µ particle size)
Mobile phase	0.1% TEA in 0.2% formic acid : acetonitrile (70 : 30)
Flow rate	1.0 mL/min
Retention time	9.38 ± 0.05 min
Detection wavelength	282 nm
Needle wash	Mobile phase
Column temperature	40ºC

4.4.2.3. Method validation using ICH Q2 (R1) guideline

4.4.2.3.1. *Linearity and range*

The calibration plotted for ACOT was found to be linear in the range of 5-30µg/mL. The regression equation was found to be y= 44304x- 28213 with regression coefficient (r^2) of 0.999. The linearity data is shown in Table 4.3 and calibration curve is shown in Fig. 4.6.

Table 4. 3 - Linearity data of ACOT

Conc. (µg/mL)	Peak Area (Mean* ±%RSD)
5	151337.7 ± 0.53
10	363314.7 ± 0.77
15	566199.7 ±0.52
20	775011.3±0.43
25	945131± 0.44
30	1182548± 0.37

*Average of three determinants

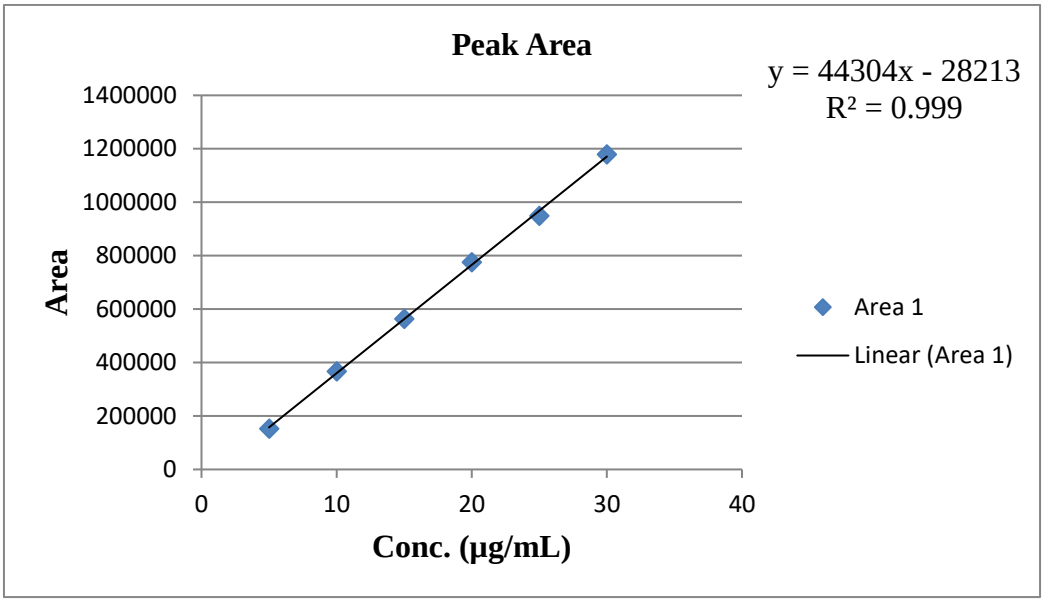


Fig. 4. 6- Calibration curve of ACOT (Peak Area versus Conc. (µg/mL)

4.4.2.3.2. *Precision*

Intra-day precision was performed by repeating the experiment three times in a day and inter-day precision was performed by repeating the experiments on three consecutive days. The average % RSD of intra-day and inter-day were found to be 0.91 and 1.11. The developed method was found to be precise (Table 4.4 and 4.5).

Table 4. 4 – Intraday precision of ACOT

Conc. (µg/mL)	Peak Area				
	Set 1	Set 2	Set 3	Mean	%RSD
5	150114	151610	153817	151847	1.22
10	352541	348410	356669	352540	1.17
15	568471	565475	559373	564439	0.82
20	764464	760782	768308	764518	0.49
25	945829	948727	938125	944227	0.58
30	1189078	1175043	1203113	1189078	1.18
Average				%RSD	0.91

Table 4. 5 – Interday precision of ACOT

Conc. (µg/ml)	Peak Area				
	Set 1	**Set 2**	**Set 3**	**Mean**	**%RSD**
5	150114	151836	152843	151597	0.91
10	366767	360396	373138	366767	1.73
15	568471	563829	559164	563821	0.82
20	772256	780780	763732	772256	1.10
25	945829	942937	957274	948680	0.79
30	1200857	1216857	1184857	1200857	1.33
Average				%RSD	1.11

4.4.2.3.3. *Accuracy*

Accuracy of method was determined by calculating % percent recovery of the analyte recovered. To the sample solution of 20µg/mL, standard solution of ACOT was added as 50%, 100% and 150% to give final concentrations as 30, 40, 50 µg/mL. Recovery greater than 99% indicates the developed method was accurate (Table 4.6).

Table 4. 6 – Accuracy data of ACOT

Excess drug added to analyte (%)	Theoretical Content (µg/mL)	*Amount Found (µg/mL)	Recovery (%)± SD
0	20	19.95	99.55±0.10
50	30	29.97	99.39±0.40
100	40	39.93	99.35±0.29
150	50	49.84	98.92±0.52

*Average of three determinations

4.4.2.3.4. *Limit of detection and limit of quantification*

LOD and LOQ were found to be 0.36 and 1.13 µg/mL respectively.

4.4.2.3.5. *Robustness*

For robustness study, slight changes were % of organic in mobile phase and flow rate, wavelength, column oven temperature. The results were expressed as % RSD. % RSD less than 2 indicated that the developed method was robust (Table 4.7).

Table 4. 7 – Robustness data of ACOT

Parameter	Level	Area		Rt		Tailing Factor		Theoretical Plates	
		Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD
% Organic	28	577713.70	0.83	11.41	0.91	1.04	0.36	12035.33	0.03
	30	578040.30	1.03	9.63	0.17	1.04	0.19	12063.67	0.24
	32	567522.00	1.02	8.59	0.85	1.04	0.33	12042.33	0.02
Flow rate	0.9	571144.30	1.26	11.39	0.56	1.03	0.20	12039.67	0.11
	1	570358.30	1.46	9.60	0.28	1.04	0.19	12084.00	0.03
	1.1	573424.30	1.30	8.34	0.30	1.04	0.29	12042.33	0.11
Wavelength	281	578040.30	1.22	9.52	0.08	1.04	0.14	11160.00	0.41
	282	581313.70	1.35	9.50	0.76	1.04	0.09	12050.33	0.27
	283	568682.00	1.02	9.51	0.17	1.03	0.09	11076.33	0.56
Column Oven Temperature (˚C)	38	575239.03	0.90	9.53	0.12	1.04	0.54	12038.33	0.17
	40	578566.20	1.20	9.51	0.04	1.05	0.54	12168.33	0.20
	42	568119.53	1.09	9.52	0.03	1.03	0.33	12253.67	0.22

% Organic and flow rate are the critical factor in the robustness method.

4.4.2.3.6. *Specificity*

The specificity was determined from the forced degradation studies as described in section 4.4.1.5. and 4.4.2.4. where Fig. 4.18 shows ACOT peak is well separated from all degradation products formed during different stress conditions with sufficient resolution. In the forced degradation studies, for all degradation products, peak purity index was greater than single point threshold , ensures degradation peaks are pure and peaks are not co-eluting. The specificity study ensures selectivity of the developed

method which is able to separate and quantify ACOT in presence of degradation products. Peak purity data of ACOT and degradation products are shown in Table 4.8.

Table 4. 8- Peak purity data of ACOT and its degradation products

S.No.	Peaks	Rt	Peak Purity Index	Single Point threshold
1	ACOT	9.38 min	1.0000	0.9999
2	DP1	3.12 min	0.999815	0.999463
3	DP2	4.07 min	0.999968	0.999284
4	DP3	5.19 min	0.999989	0.999596

4.4.2.3.7. *Stability in sample solutions*

Stock solution of ACOT and stressed samples were prepared from standard stock solution and then stored at room temperature for 24 hrs. No additional peaks were observed which indicated stability of ACOT sample solution.

4.4.2.3.8. *System Suitability Parameters*

System suitability tests were performed on freshly prepared solution with n=6 containing ACOT. The results of system suitability parameters are shown in Table 4.9. Peak purity data of ACOT is shown in Table 4.8 and peak purity curve is shown in Fig.4.7.

Table 4. 9 – System suitability parameters of ACOT

Parameters	Data Obtained
Retention Time (min ± SD)	9.38 ± 0.05
Tailing Factor ± SD	1.04± 0.04
Theoretical Plates ± SD	12098 ± 21.36

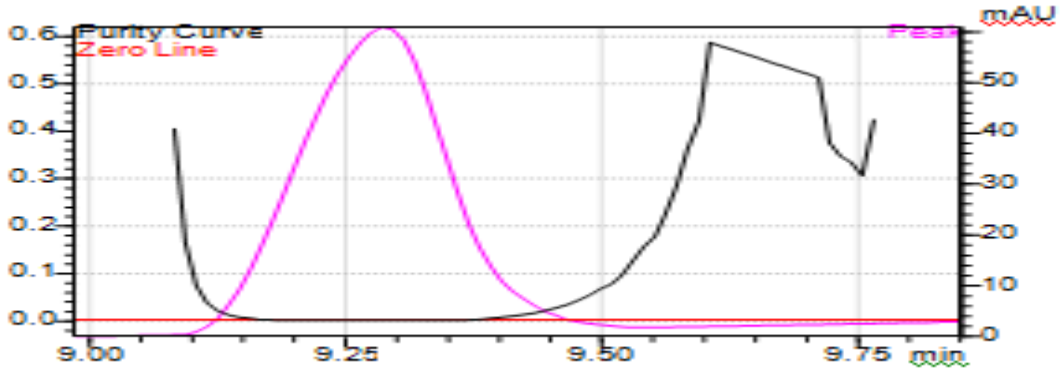


Fig. 4. 7- Peak purity of ACOT

4.4.2.4. Stress Degradation studies

4.4.2.4.1. Acid degradation – Slight degradation (0.92%) was observed when ACOT was subjected to 1 M HCl at 100ºC for 3 hrs with the formation of one degradation product DP3 at retention time of 5.30 min (Fig. 4.8).

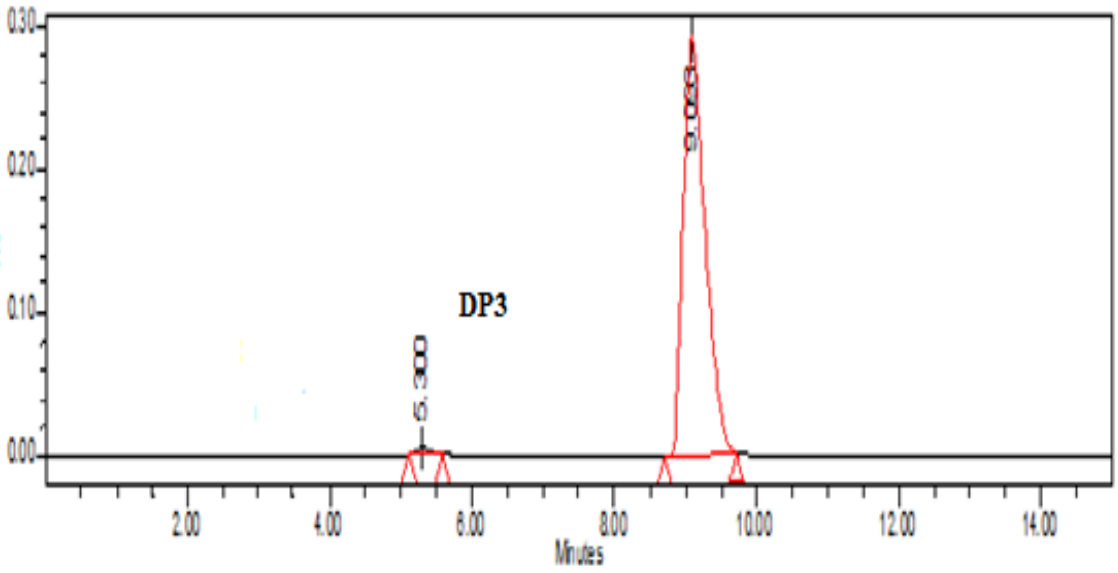


Fig. 4. 8– Chromatogram of acid degradation (API)

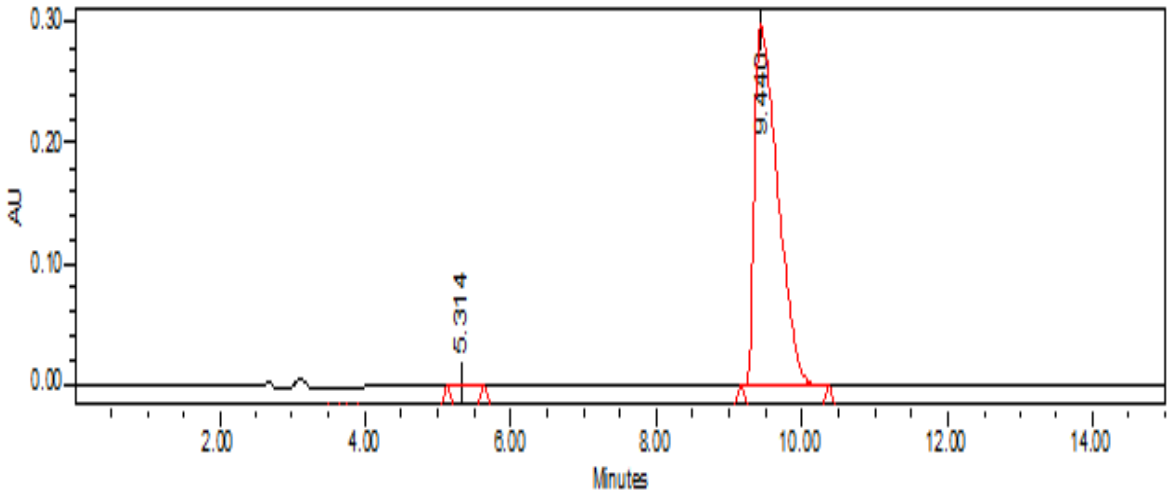


Fig. 4. 9 – Chromatogram of acid degradation (formulation)

4.4.2.4.2. Alkaline degradation – significant degradation(15.7%) was observed when ACOT was subjected to 0.5 M NaOH at 100ºC for 3 hrs with the formation of three degradation products DP1, DP2 and DP3 at retention time of 3.59 , 4.07, 5.12 min respectively (Fig. 4.10).

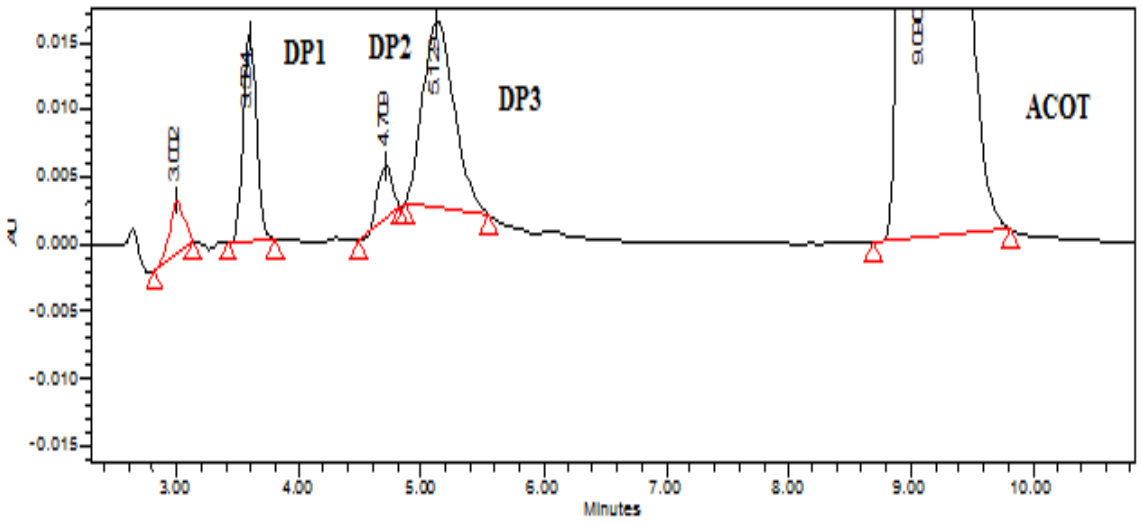


Fig. 4. 10 – Chromatogram of alkaline degradation (Zoomed View - API)

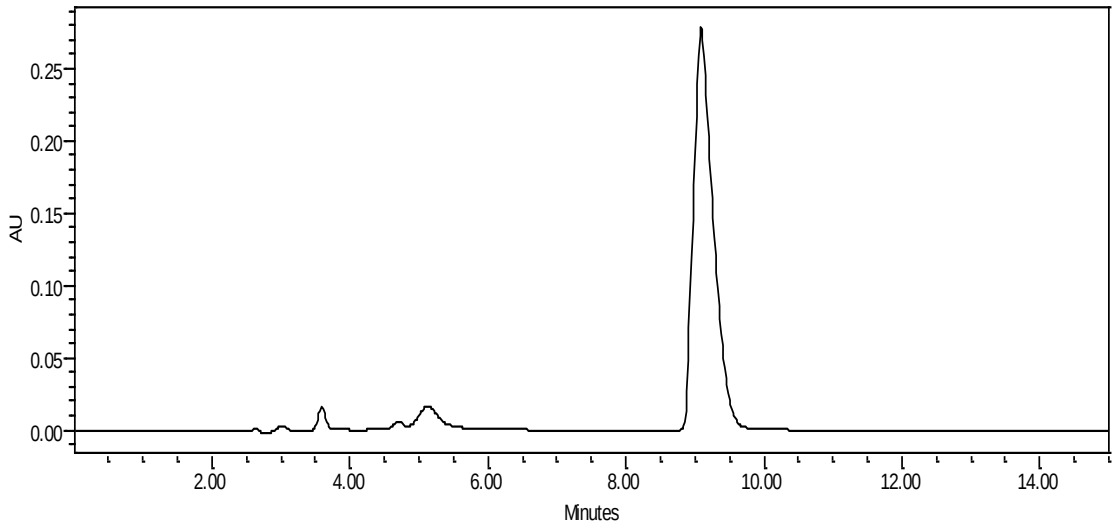


Fig. 4. 11 - Chromatogram of alkaline degradation (Full View- API)

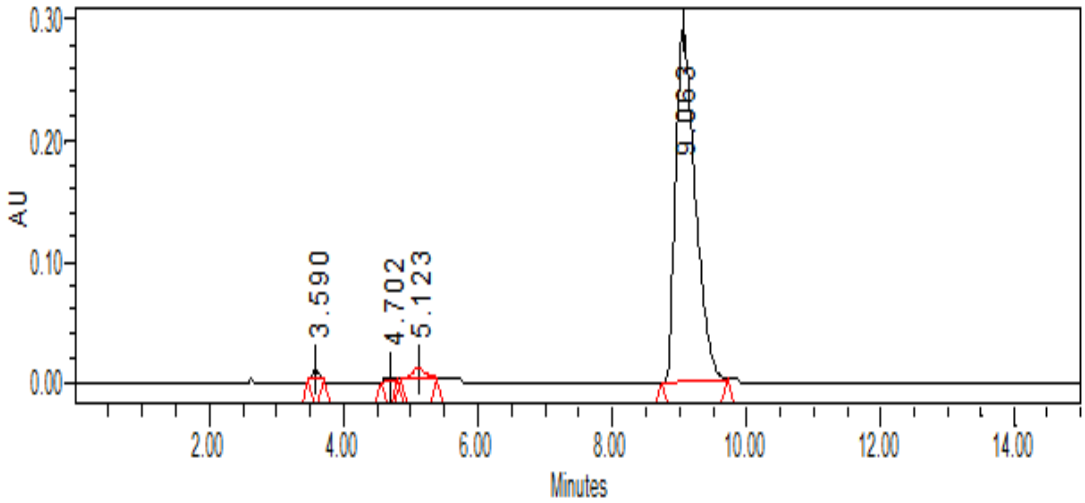


Fig. 4. 12 -Chromatogram of alkaline degradation (formulation)

4.4.2.4.3. Neutral hydrolysis degradation- No additional peak was observed when ACOT was subjected to neutral hydrolysis at 100ºC for 6 hrs (Fig. 4.13).

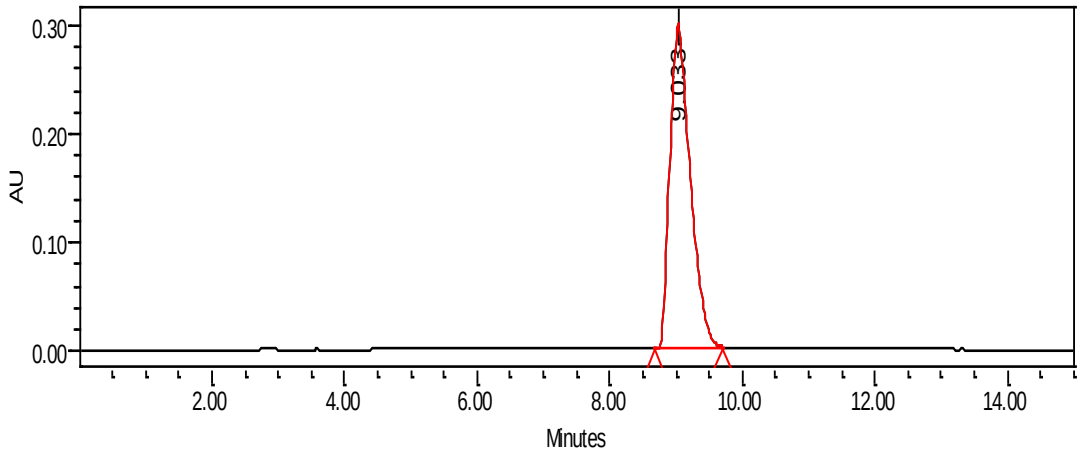


Fig. 4. 13 – Chromatogram of neutral hydrolysis

4.4.2.4.4. Oxidative degradation- No additional peak was observed when ACOT was subjected to 6% hydrogen peroxide at room temperature for 48 hrs (Fig. 4.14).

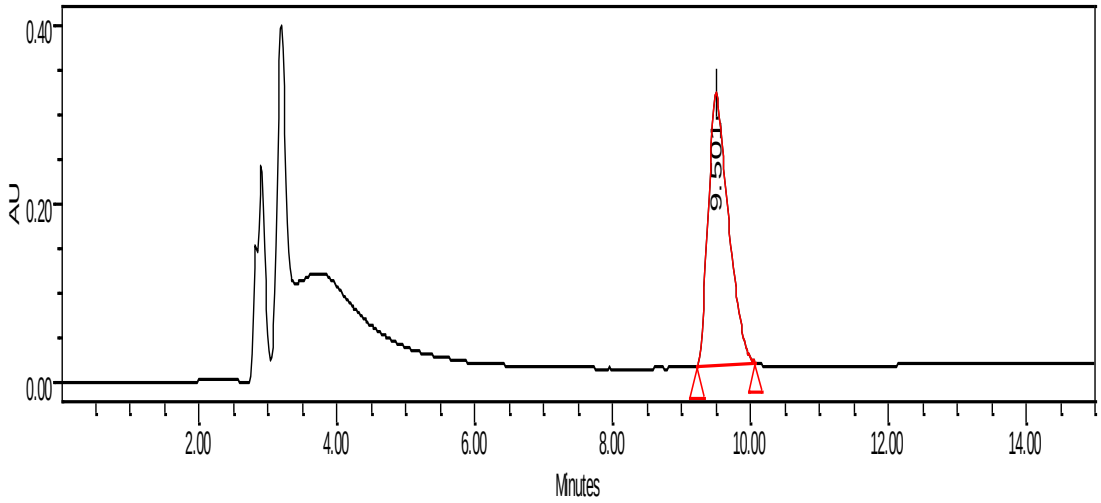


Fig. 4. 14 – Chromatogram of oxidative degradation

4.4.2.4.5. Dry heat degradation- No degradation was observed when ACOT was subjected to thermal degradation at 80ºC for 11 days (Fig. 4.15).

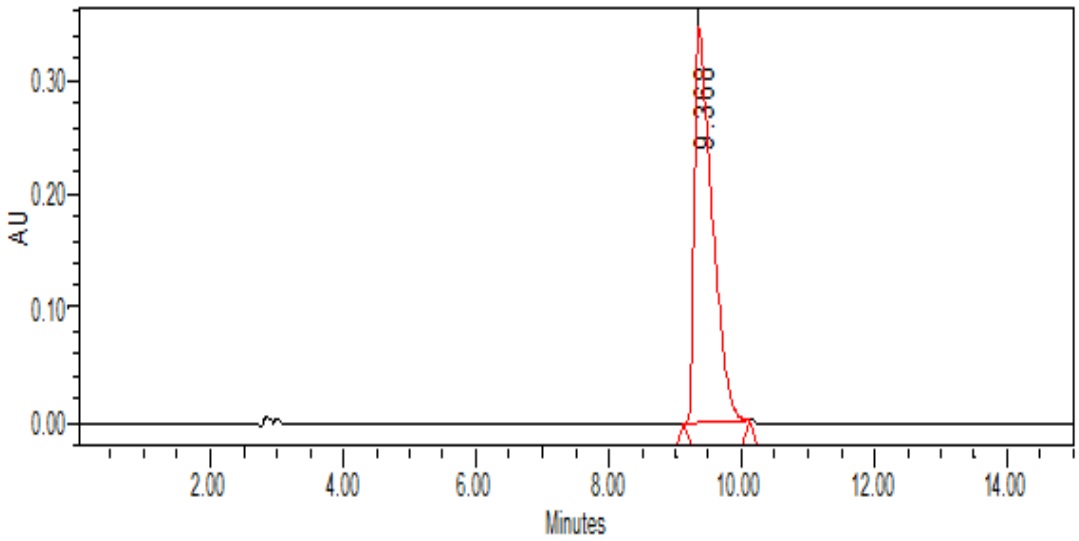


Fig. 4. 15 - Chromatogram of thermal degradation

4.4.2.4.6. Photolytic degradation -No degradation was observed when ACOT was subjected to photolytic condition (dry and solution) for 11days (Fig. 4.16 and 4.17).

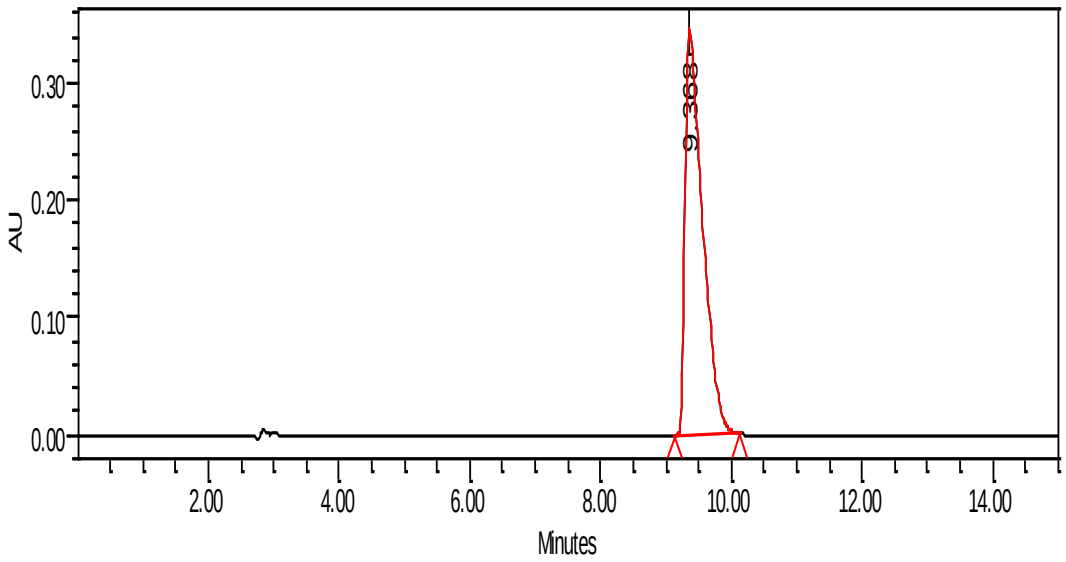


Fig. 4. 16 - Chromatogram of photolytic degradation (Dry)

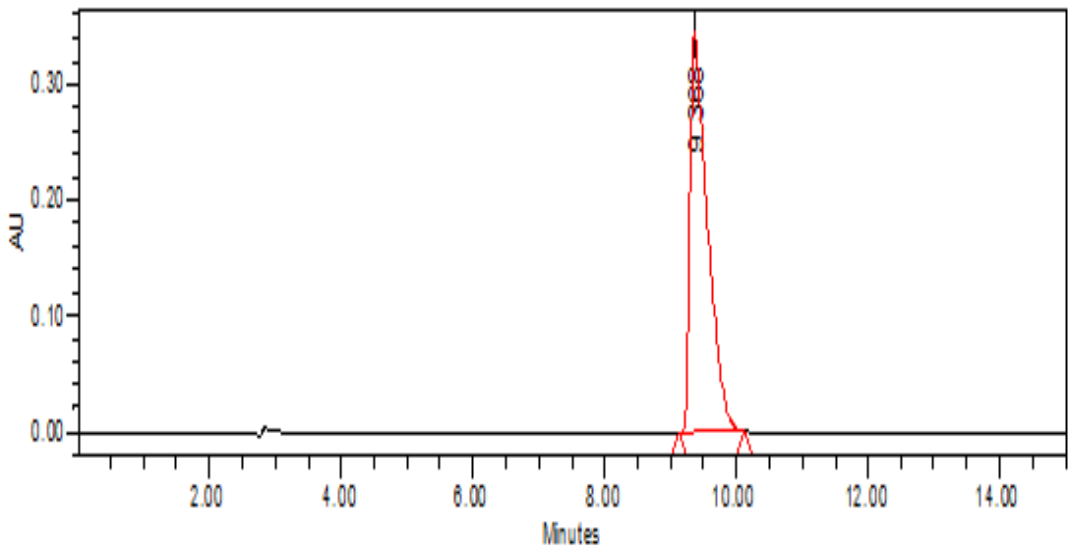


Fig. 4. 17- Chromatogram of photolytic degradation (Solution)

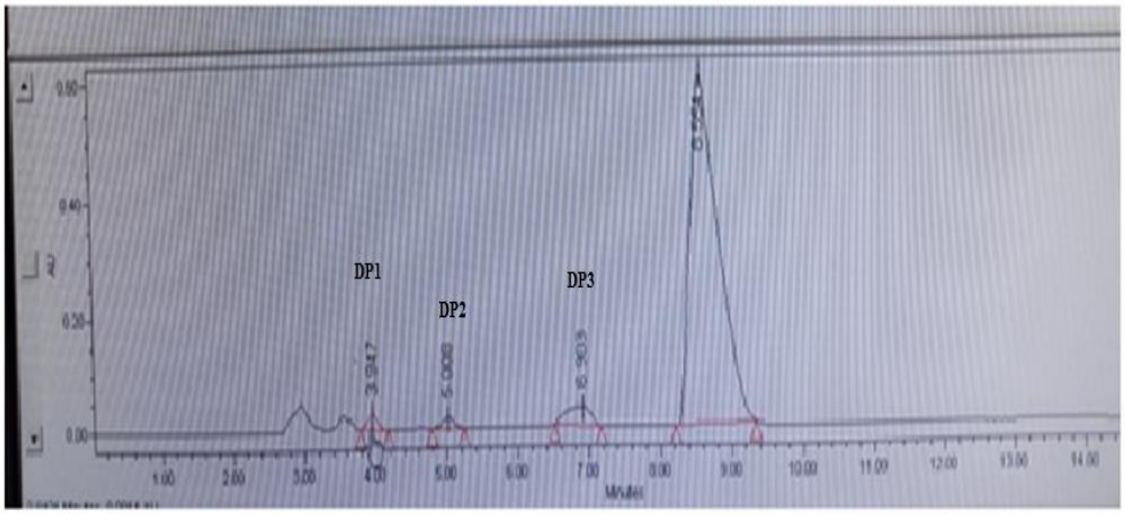


Fig. 4. 18 – Chromatogram of combined degradation products of all stressors

Table 4. 10 - Summary of forced degradation study of ACOT

Stressor	Conditions	RT of degradation products	%Degradation (API)	% Degradation (Formulation)
Acid	1 M HCl 100℃ for 3 hours	5.36 (DP3)	0.92%	0.71%

Alkaline	0.5 M NaOH 100ºC for 3 hours	3.59 (DP1) 4.07 (DP2) 5.12 (DP3)	15.7%	14.2%
Neutral hydrolysis	100ºC for 6 hours	--	--	--
Oxidation	6% H_2O_2 RT for 48 hours	--	--	--
Thermal	Dry at 80℃ for 8 days	--	--	--
Photolytic	5382 Lux and 144 $UVcm^{-2}$ for 11 days	--	--	--

4.4.2.5. Applicability of the developed method for the analysis of formulation

Forced degradation study was performed on formulation. The conditions were same as mentioned for API and were analyzed in the same way as that of API. The degradation products were separated. Minor variation was observed in the degradation of API and formulation as shown in Table 4.10.

4.4.3. DISCUSSIONS

Maximum absorption wavelength of 282 nm was selected by scanning in the range from 200-400 nm. Various trials were conducted for optimization of chromatographic procedure. With mobile phase ratio of water and methanol, ACOT was not eluting. Initially method was optimized with 0.1 % formic acid and methanol. During stability indicating method development in alkaline condition, one of degradation product was co-eluted with ACOT. Separation of degradation product from ACOT was achieved on C 8 column using mobile phase 0.1% triethylamine in 0.2% formic acid and acetonitrile in ratio of 70:30. Significant degradation (15.7%) was obtained in alkaline condition with the formation of three degradation products. Slight degradation (0.92%) was obtained in acidic condition with the formation of one degradation

product. ACOT was stable in oxidative, neutral hydrolytic, thermal and photolytic conditions as there wasn't any additional peak in the chromatogram and peak height of ACOT was not reduced. The developed method was validated as per ICH guidelines with respect to linearity, precision, accuracy, limits of detection and robustness. Good correlation was obtained between peak area and concentration of ACOT in the range of 5µg/mL to 30 µg/mL with regression coefficient r^2 0.999. % RSD for intra-day and inter-day precision was less than 2. % Recovery was found to be in the range of 98.92%-99.35%. % RSD for robustness studies was less than 2. This indicated that the developed method is precise, accurate and robust to small changes in the experimental conditions.

4.5. SECTION - B

ISOLATION AND CHARACTERIZATION OF MAJOR DEGRADATION PRODUCTS OF ACOTIAMIDE HCL TRIHYDRATE

4.5.1. EXPERIMENTAL

4.5.1.1. Chemicals and Reagents

Chemicals and reagents used in the present section are same as those mentioned in 5.4.1.1.

4.5.1.2. Equipments and chromatographic conditions

Preparative High Performance Liquid Chromatography (HPLC):

Preparative HPLC system used in the study was Shimadzu LC-20 AP pump fitted with SPD 20A detector.

Separation was performed on Phenomenex Luna C-8(2) column with 250x50 mm, 100 Å, 10µ in diameter. Detection was performed at 282 nm. Flow rate was maintained at 60mL/min. Samples were injected through Rheodyne 7725 injector valve.

Mobile phase comprised of 0.1% triethylamine with 0.2% formic acid: acetonitrile and gradient programme was run as: time (min)/%of mobile phase (acetonitrile) – 20/20, 21/20, 25/40, 35/20.

4.5.1.3. Enrichment of alkaline degradation samples

1g of ACOT was weighed accurately and transferred to 50mL of volumetric flask. To this was added 30 mL of water followed by 10 mL of 1 M NaOH. This solution was heated at 100ºC for 8 hours and after cooling, neutralized by 1 M HCl. The remaining volume was made up to the mark 50 mL with water. Fours similar batches were processed in a similar manner to collect sufficient quantity of degradation products DP2 and DP3.

4.5.1.4. Analysis of degradation samples by analytical HPLC

The separated alkaline degradation products were accessed for their purity after analyzing a diluted sample by analytical HPLC. The degradation product (DP2) was 5.2% as suggested by area normalization and appeared at retention time 8.11 min whereas DP3 was 18.7% as per area normalization with a retention time of 15.52 min (Fig. 4.19).The retention time of ACOT was 26.09 min.

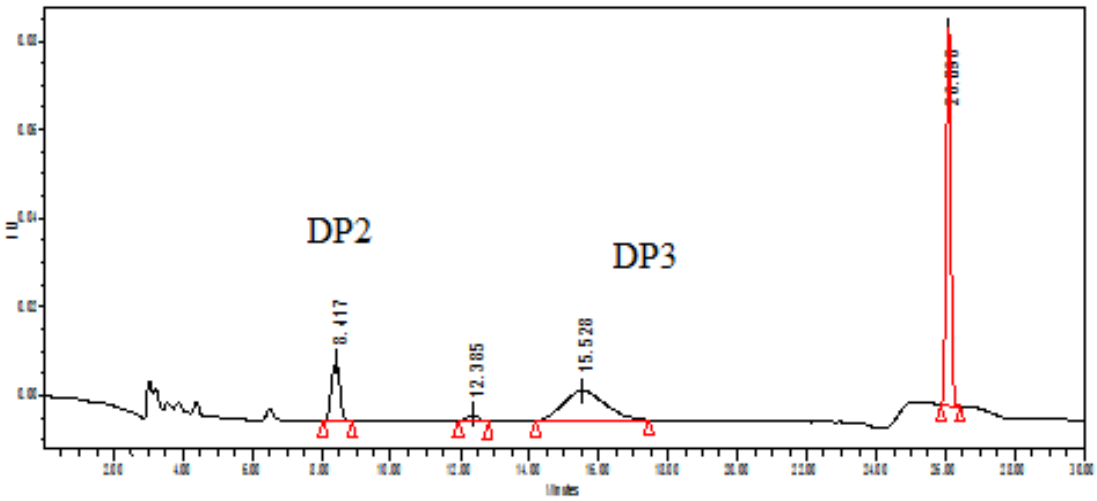


Fig. 4. 19 – Chromatogram of alkaline degradation for isolation

4.5.1.5. Isolation of degradation products by preparative HPLC

Degradation products DP2 and DP3 were separated by preparative HPLC. Fractions of DP2 and DP3 at their respective retention time were collected. Acetonitrile in the both the fractions was removed using rotary evaporator.

DP3 – The residue obtained after removal of acetonitrile, was filtered and analysed by analytical HPLC to confirm the retention time. The resulting product was DP3 obtained with 99.8% purity (Fig. 4.21.).

DP2- The aqueous layer after removal of organic layer acetonitrile, was further analysed. Filtrate was containing DP2 along with some percentage of DP3 and ACOT. This filtrate was further purified by using mobile phase water and acetonitrile followed by mobile phase ammonium acetate buffer pH 6.5 and acetonitrile. Solution was subjected to rotary evaporator to remove acetonitrile. The remaining solution was lyophilized. DP2 was obtained with colorless solid with purity of 99.2% (Fig. 4.20.).

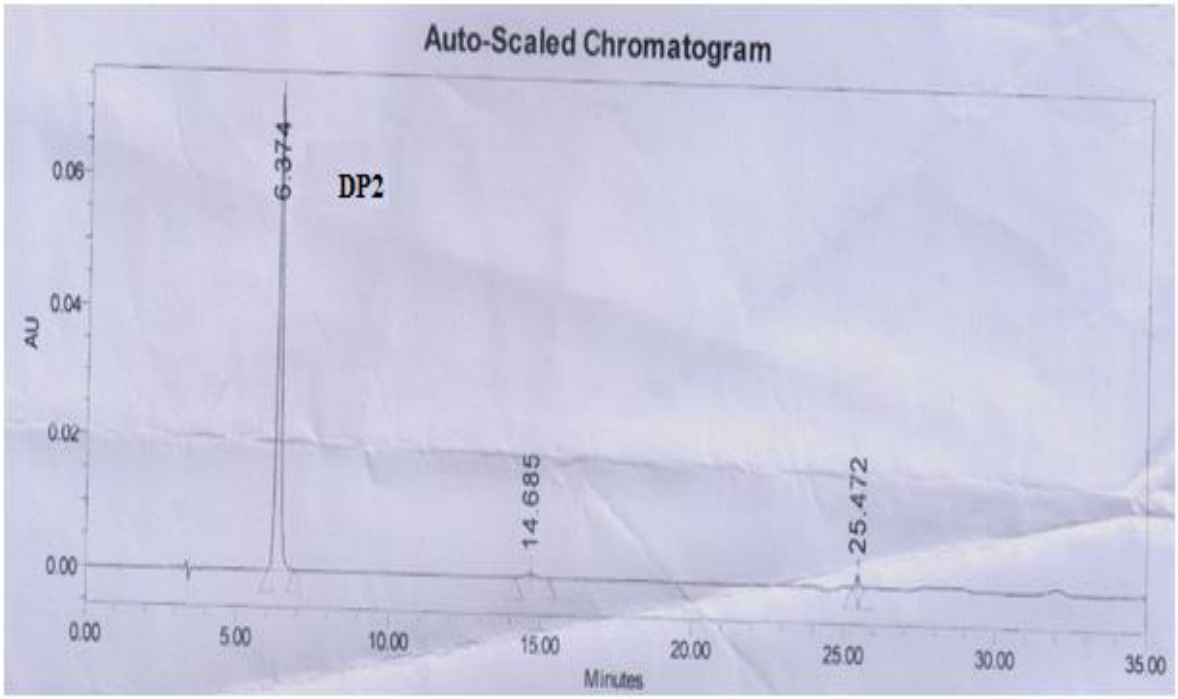


Fig. 4. 20 - Analytical Chromatogram of DP2 after isolation and purification

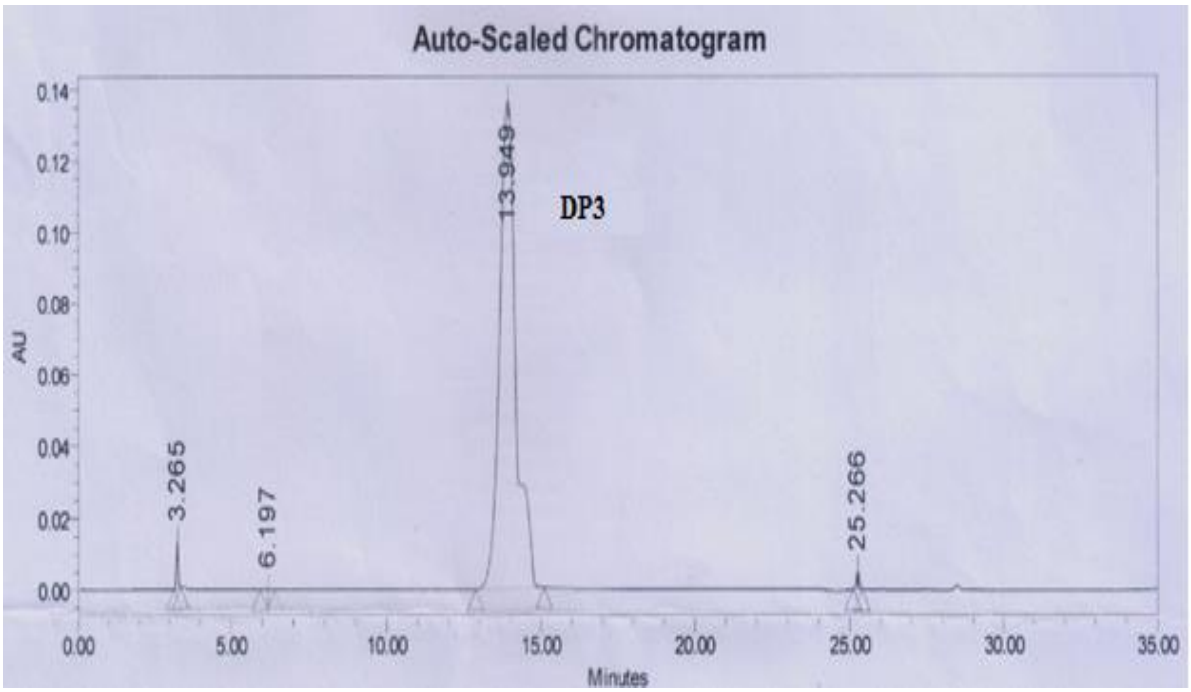


Fig. 4. 21 - Analytical chromatogram of DP3 after isolation and purification

4.5.2. RESULTS AND DISCUSSIONS

4.5.2.1. Identification of degradation products

IR, MS and NMR spectra were obtained for ACOT, DP2 and DP3

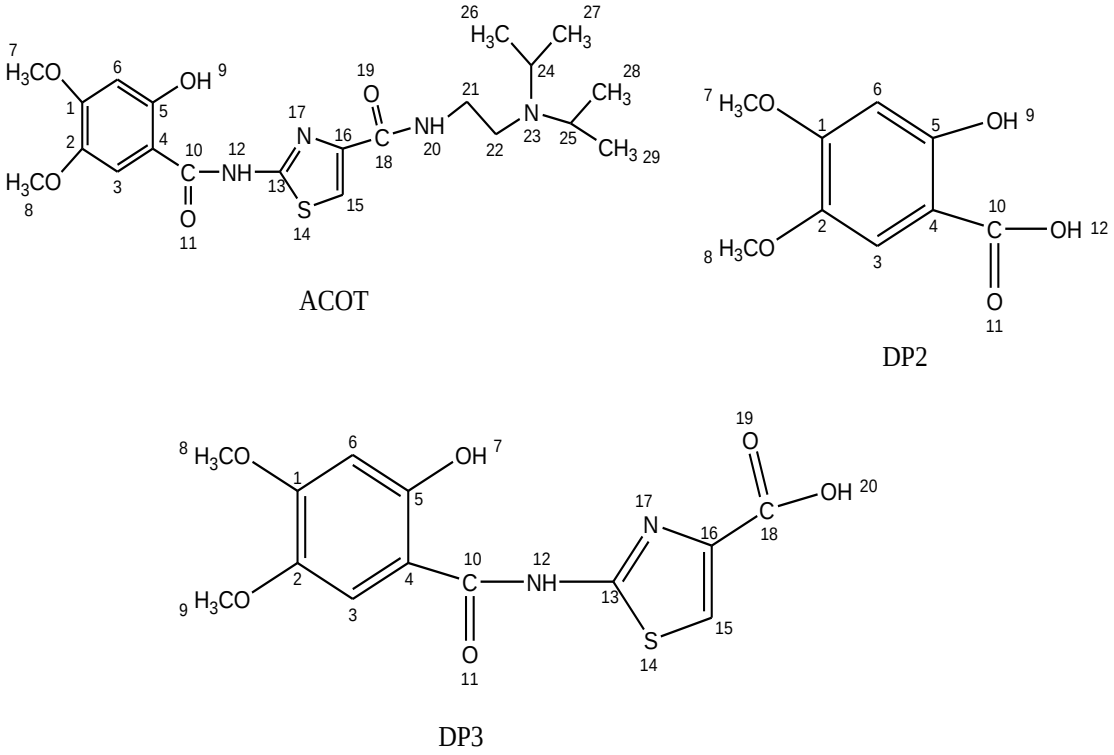


Fig. 4. 22 - Structure of ACOT, DP2 and DP3 with assigned numbers

4.5.2.1.1. Spectral data of ACOT

Mass spectra

An ESI-MS/MS spectrum of ACOT is provided in Fig.4.23 .ESI MS/MS of spectra shows protonated m/z 451 and main fragments at m/z 349.57, 271.10, 229.14 and 170.04. Formation of these fragments can be attributed due to the loss of diisopropyl amine. The loss of 2,3,dimethoxy4-hydroxy benzene carbonyl is followed by loss of isopropyl group and then loss of diisopropyl amino group (Fig. 4.24).

NMR spectra

The integration data of ^{1}H NMR spectra of ACOT indicates presence of 12 H suggesting four methyl groups at 1.4 ppm, two methine groups at 3.2 ppm, two methylene groups at 3.5 ppm and ^{13}C NMR four methyl groups are indicated at 18.1 and 19.2 ppm and methylene groups at 38 and 46 ppm. Methoxy groups are indicated

by the presence of peak at 3.8 and 3.9 ppm and in ^{13}C NMR and DEPT at 56.1 ppm. Presence of two –NH protons of secondary amide are shown by the presence of peak at 8.8 and 11.8 ppm which are absent in D_2O exchange .In ^{13}C NMR carbonyl groups are shown at 158 and 162 ppm. Presence of –OH group is indicated by broad peak at 9.5 ppm which is absent in D_2O exchange (Table 4.11).

I.R. Spectra

I.R. spectra of ACOT indicate the presence of two amide groups at 3481 and 3298 cm^{-1}, presence of aromatic ring at 3098 cm^{-1}. Carbonyls of two amide groups are indicated at 1639 and 1564 cm^{-1} (Fig. 4.25, Table 4.12).

4.5.2.1.2. Characterization of DP2

Mass spectra

Mass spectra of DP2 shows molecular ion peak at m/z 198 in negative ion mode. DP2 is 252 m/z less than ACOT (Fig. 4.26).

NMR spectra

Two methoxy groups are indicated at 3.76 and 3.86 ppm in ^{1}H NMR and at 55 and 57 ppm in ^{13}C NMR. Two aromatic protons are shown by the peaks at 6.6 and 7.2 ppm in ^{1}H NMR spectra. There is a broad peak covering two –OH groups which are absent in D_2O exchange. There is one carbonyl group which is shifted to downfield 172 ppm in ^{13}C NMR spectra. Four methyl groups, two methylene groups, two isopropyl groups, two secondary amide (-CONH) and thiazole groups peaks are absent in DP2. Formation of additional –OH group and shifting of carbonyl group to downfield indicated that hydrolysis has taken place at amide group at dimethoxy benzene moiety resulting in the formation of DP2 (Table 4.13).

IR spectra

In DP2, two amide groups at 3481 and3298 cm^{-1}, one of carbonyl group of amide of ACOT are absent but a new peak of carboxylic acid appears. Presence of carboxylic acid is indicated by formation of peak (-OH bending) at 1392 cm^{-1} and carbonyl peak of carboxylic acid at 1643cm^{-1} (Table 4.12, Fig. 4.27).

Based on the above, it can be inferred that DP2 is formed from ACOT by alkaline hydrolysis between positions 10 and 12 position of ACOT. DP2 is characterized as 2-hydroxy-4, 5-dimethoxy benzoic acid.

4.5.2.1.3. Characterization of DP3

Mass spectra

Mass spectra of DP3 shows molecular ion peak at m/z 323 in negative ion mode. DP3 is 127 m/z less than ACOT (Fig. 4.34).

NMR spectra

NMR spectra of DP3 indicates absence of four methyl groups at 1.44 ppm, two methylene groups at 3.2. and 3.5 ppm. and two methine groups at 3.5 ppm as indicated by proton NMR. There is absence of one amide group at 9.5 ppm. Formation of additional –OH peak is shown by broad peak at 12 ppm which is absent in D_2O exchange.^{13}C NMR spectra of DP3 indicates absence of four methyl groups at 18.1 and 19.2 ppm , two methylene groups at 38, 46 ppm , two methine groups at 53.5 ppm. There is absence of carbonyl at 158 ppm which is shifted to downfield at 164 ppm which indicates that amide has been converted to carboxylic acid (Table 4.14).

I.R. spectra

In I.R. spectra of DP3, presence of secondary amide –NH stretching is indicated at 3248cm^{-1}, there is absence of secondary amide stretching which is present in ACOT. Compared to ACOT, one of the carbonyl group is shifted to 1678cm^{-1} and there is appearance of –OH bending at 1390cm^{-1}(Fig 4.35 and Table 4.12) .

From the above, it can be concluded that DP3 is formed from ACOT by alkaline hydrolysis. Hydrolysis has taken place between 18 and 20 position of amide group in ACOT. DP3 is characterized as 2-[(2-hydroxy-4, 5-dimethoxybenzoyl) amino]-1, 3-thiazole-4-carboxylic acid.

Table 4. 11 – NMR assignments of ACOT

ACOT					
Position	**^{1}H (Fig.4.28)**	**Chemical Shift**	**Position**	**^{13}C Chemical shift (Fig. 4.29)**	**DEPT (Fig. 4.30)**
12	1H	11.8, s ,- NH absent in D_2O	10	162	carbonyl
9	-OH	9.5, Broad peak absent in D_2O	13	160	Quaternary carbon
20	1H	8.8, s, -NH absent in D_2O exchange	18	158	carbonyl
3,6	2H	8.0, 7.6,s	1,5	154,152	Quaternary carbon
15	1H	6.8,s	16	144	Quaternary carbon
7,8	6H	3.9, 3.8 ,s	2	142	Quaternary carbon
21,22	4H	3.5, m	15	118	Aromatic-CH-
24,25	2H	3.2,m	4	116	Quaternary carbon
26,27,28,29	12H	1.4,m	3,6	113,102	Aromatic-CH-
			7,8	56.1	$-CH_3$
			24,25	53.5	-CH
			22, 21	46, 38	$-CH_2$
			28,29	19.2	$-CH_3$
			26,27	18.1	$-CH_3$

Table 4. 12– I.R. spectra of ACOT, DP2 and DP3

ACOT		DP2		DP3	
Wave number (cm^{-1})	Assignments	Wave number (cm^{-1})	Assignments	Wave number (cm^{-1})	Assignments
3481	Stretching CONH	2945	Aromatic C-H Stretch	3248	Stretching CONH
3298	Stretching CONH	2829	Stretching Methyl	2972	Aromatic stretch
3098	Aromatic stretch	1643	Carbonyl Acid	2749	Stretching Methyl
2953	Stretching CH_3	1564	Absent		
2673	Stretching CH_2	1510	Aromatic C=C Stretch	1678	Carbonyl Acid
1639	Carbonyl amide I	1456		1641	Carbonyl Amide
1564	Carbonyl amide II	1392	-OH Bending	1612	Bending N-H
1519	Aromatic C=C ring stretching	1357	C-O Stretch	1516	Aromatic C=C stretch
1492	C-N Stretch	1205	C-O-C Stretch	1431	
1267				1390	-OH bending
1207	C-O-C Stretch			1213	C-O-C stretch
1122	C-N stretch			1163	C-N Stretch
1080	C-O Stretch			775	C-S linkage
781	C-S linkage			702	Out of plane N-H bending
692	Out of plane N-H wagging				

Table 4. 13 – NMR assignments of DP2

DP2					
Position	**^{1}H (Fig. 4.31)**	**Chemical Shift(δ ppm)**	**Position**	**^{13} C (Fig. 4.32)**	**DEPT 135 (Fig. 4.33)**
9,12	-OH	12.5,s, broad peak	10	172	Carbonyl
Aromatic 3,6	2H	7.2, 6.6, s	1,2	158, 160	Quaternary carbon
7,8	6H	3.86, 3.76 s	5	141	Quaternary carbon
			3	110	-CH-
			4	104	Quaternary carbon
			6	100	-CH-
			7,8	55,57	$-CH_3$

Table 4. 14 – NMR assignments of DP3

DP3					
Position	**^{1}H(Fig. 4.36)**	**Chemical Shift(δ ppm)**	**Position**	**^{13} C (Fig. 4.37)**	**DEPT (Fig. 4.38)**
7,12, 20	-OH	12, s, broad peak	10,18	160,164	Carbonyl
15	1H	8.1,s	13	158	Quaternary carbon
3,6	2H	7.8,6.6,s	1,5	154,152	Quaternary carbon
8, 9	6H	3.8,s	2	142	Quaternary carbon

	16	144	Quaternary carbon
	3	119	Aromatic -CH-
	15	110	Aromatic -CH
	4	104	Quaternary carbon
	6	100	Aromatic -CH-
	8, 9	58.2	$-CH_3$

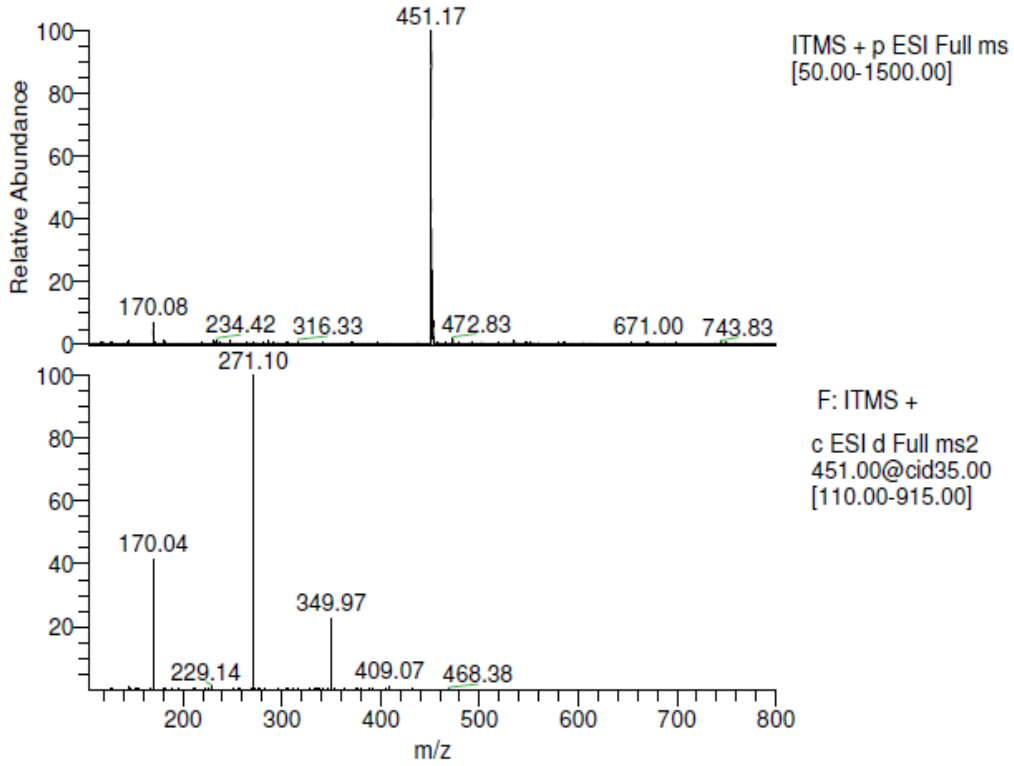


Fig. 4. 23 - ESI-MS/MS spectra of ACOT

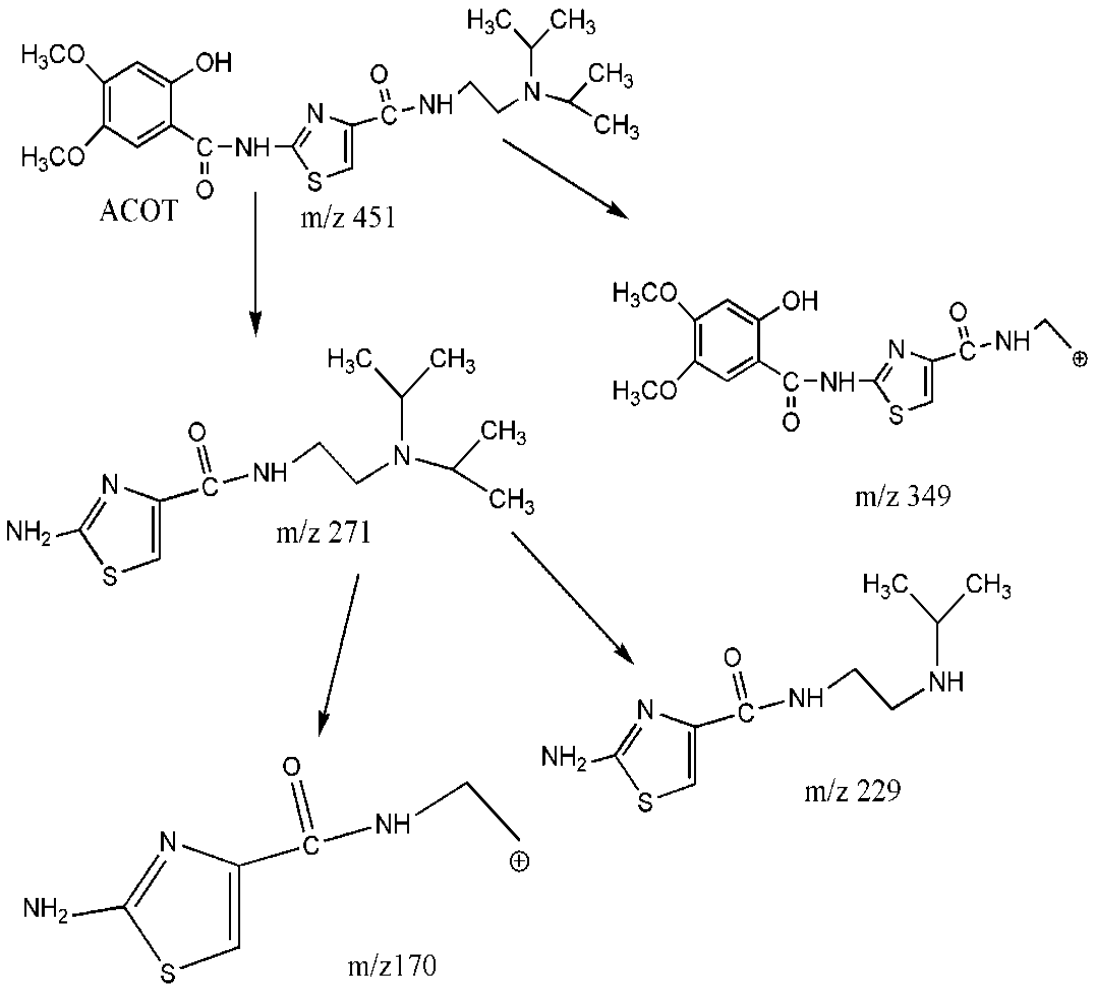


Fig. 4. 24 - Fragmentation pathway of ACOT

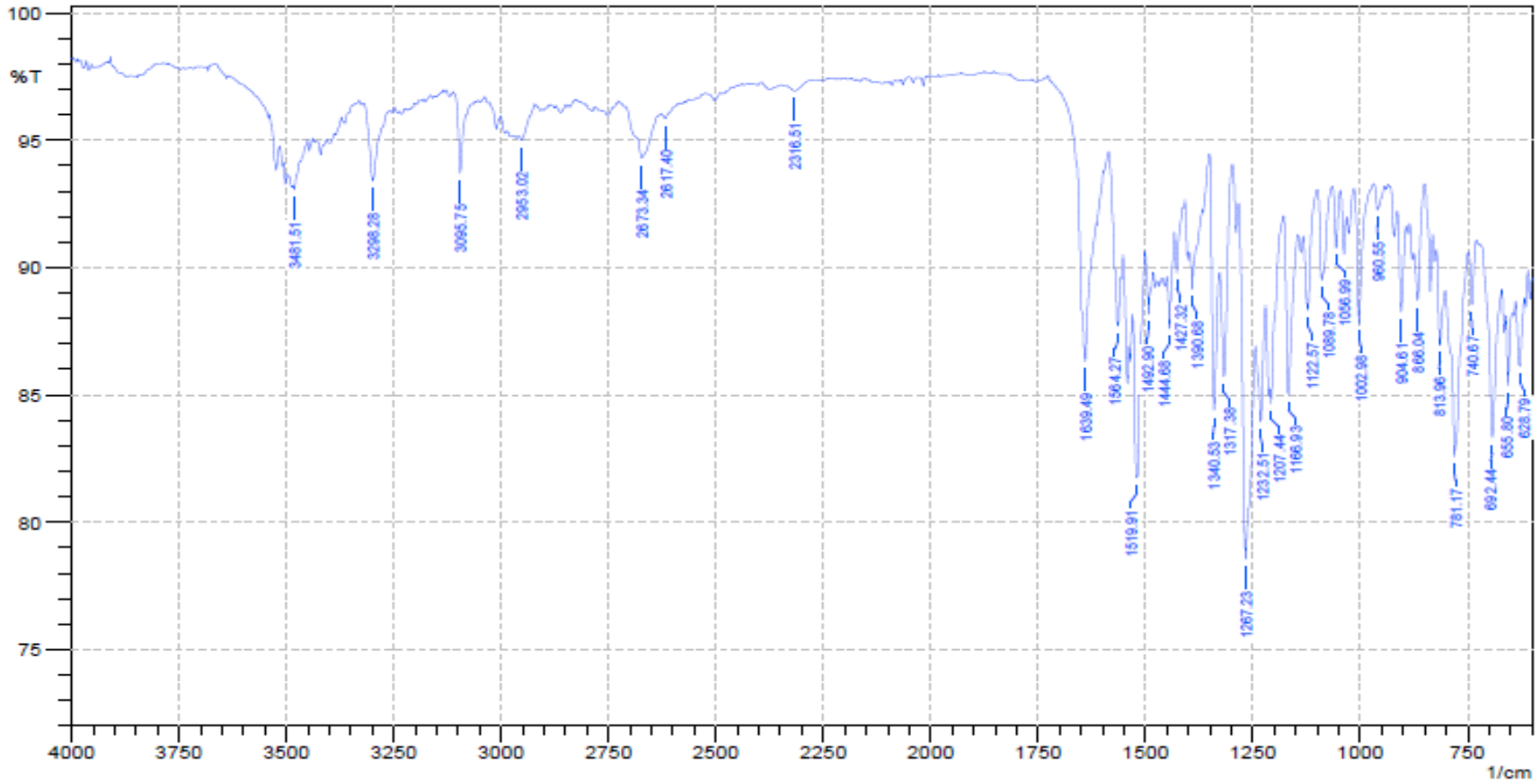


Fig. 4. 25- I.R. spectra of ACOT

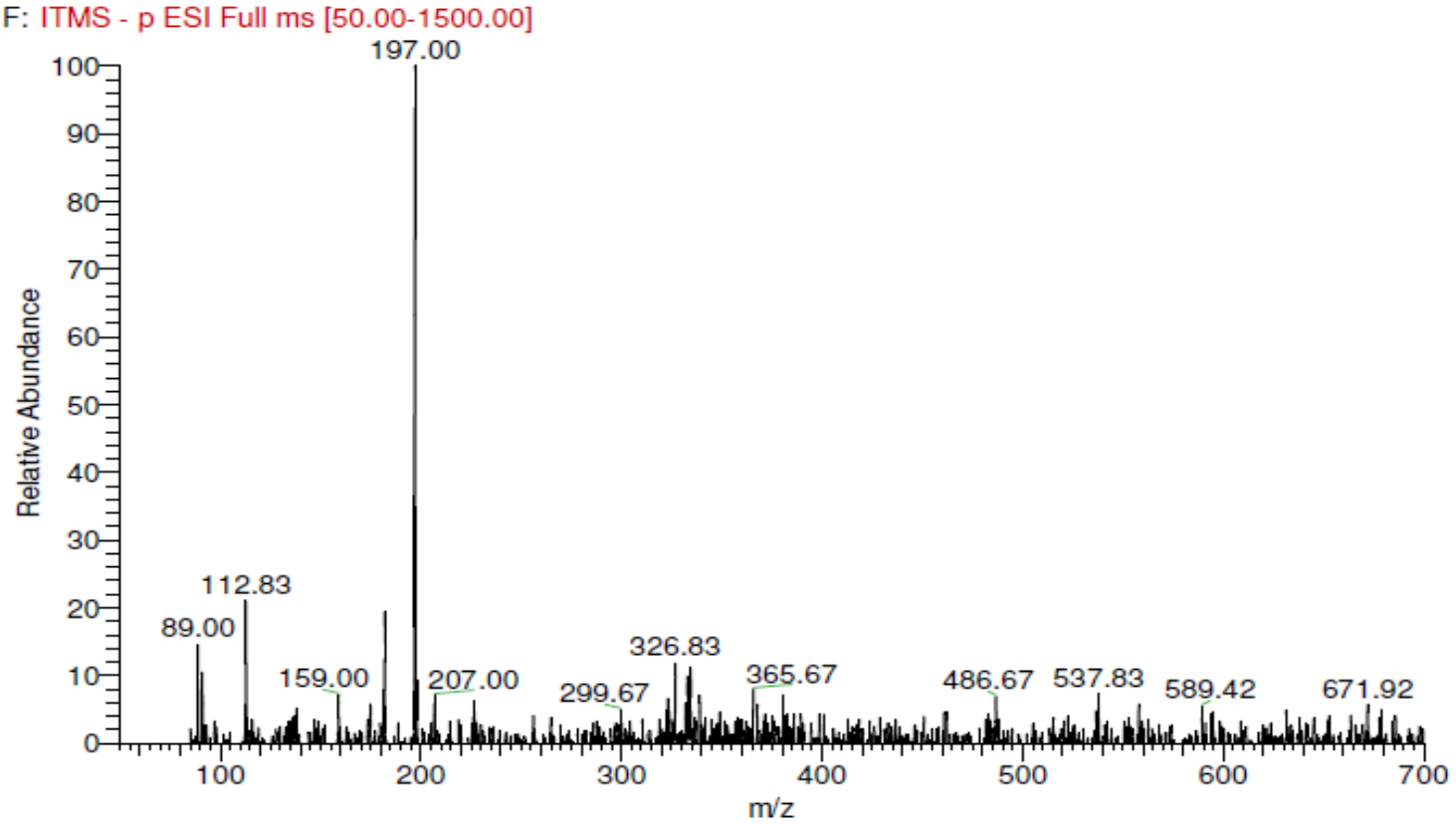


Fig. 4. 26 - ESI-MS spectra of DP2

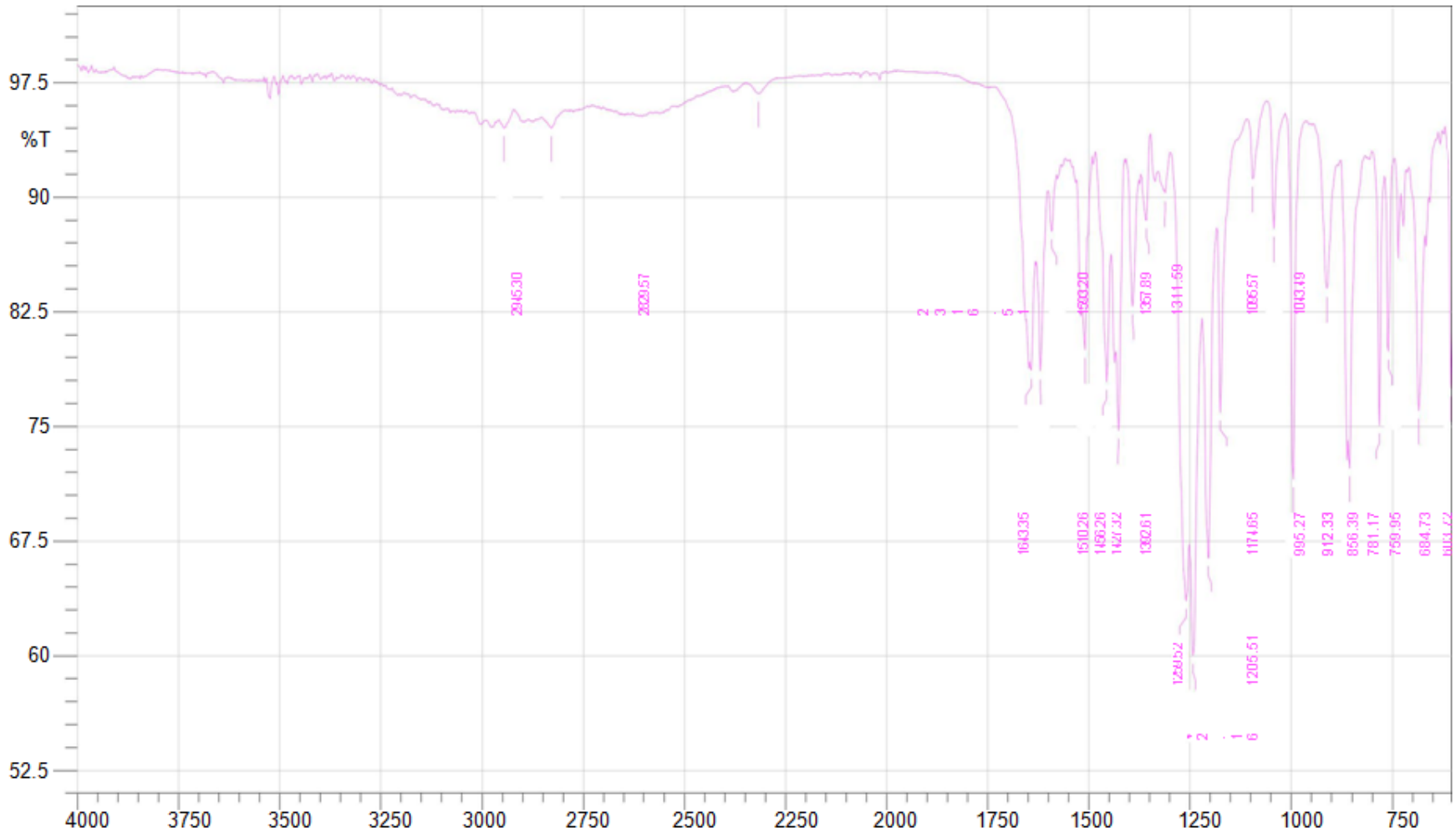


Fig. 4. 27- I.R. spectra of DP2

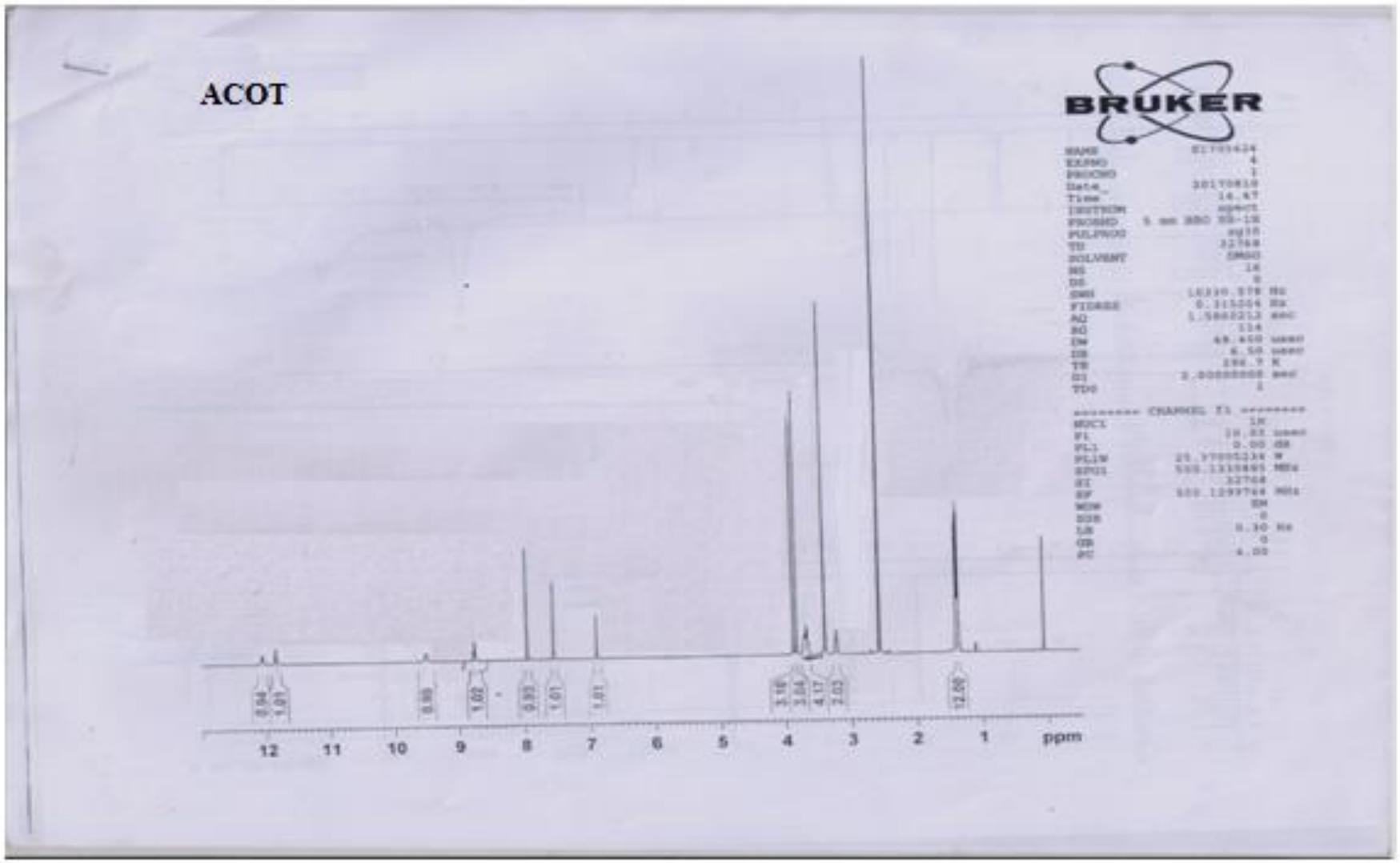


Fig. 4. 28 -^{1}H NMR spectra of ACOT

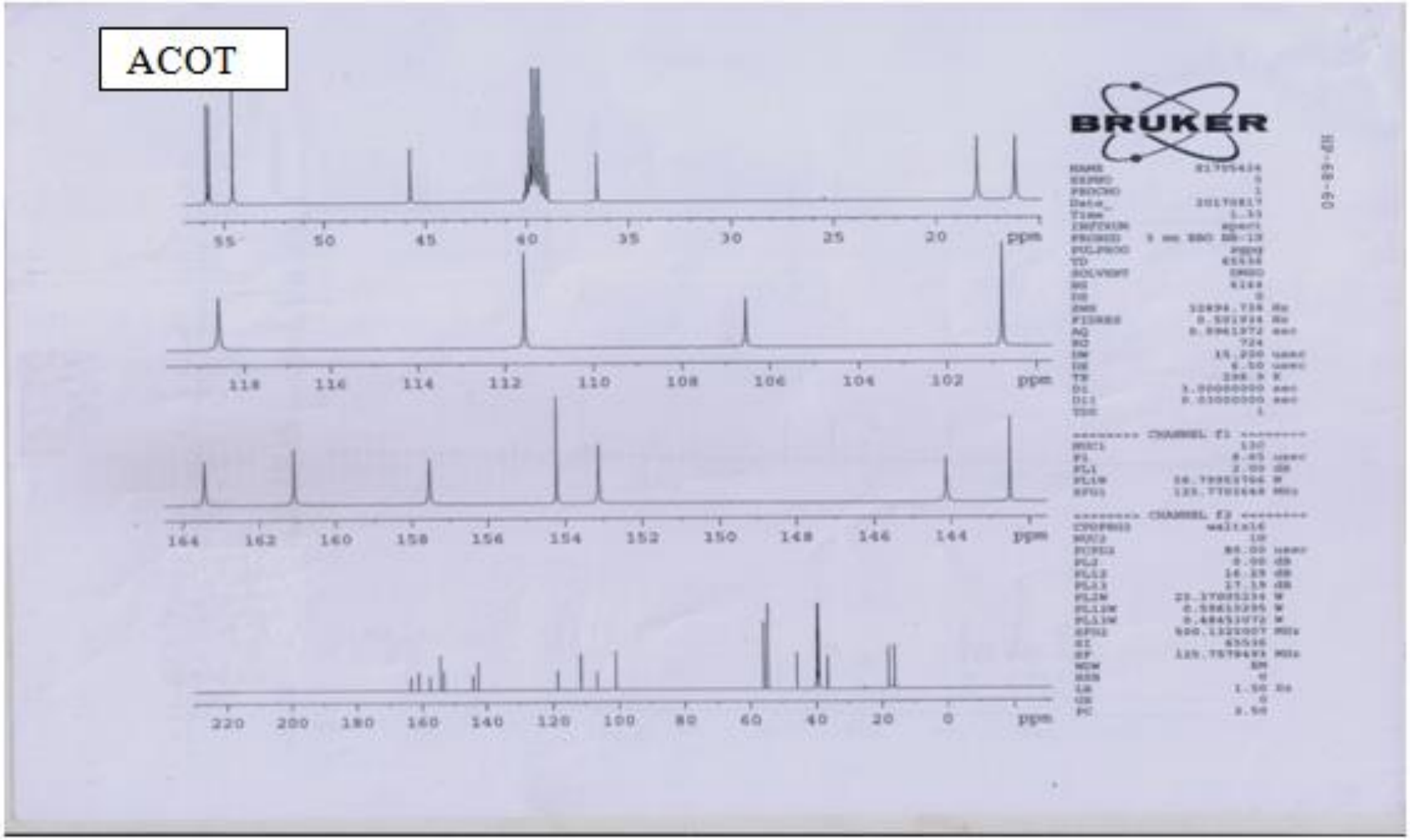


Fig. 4. 29 – ^{13}C NMR spectra of ACOT

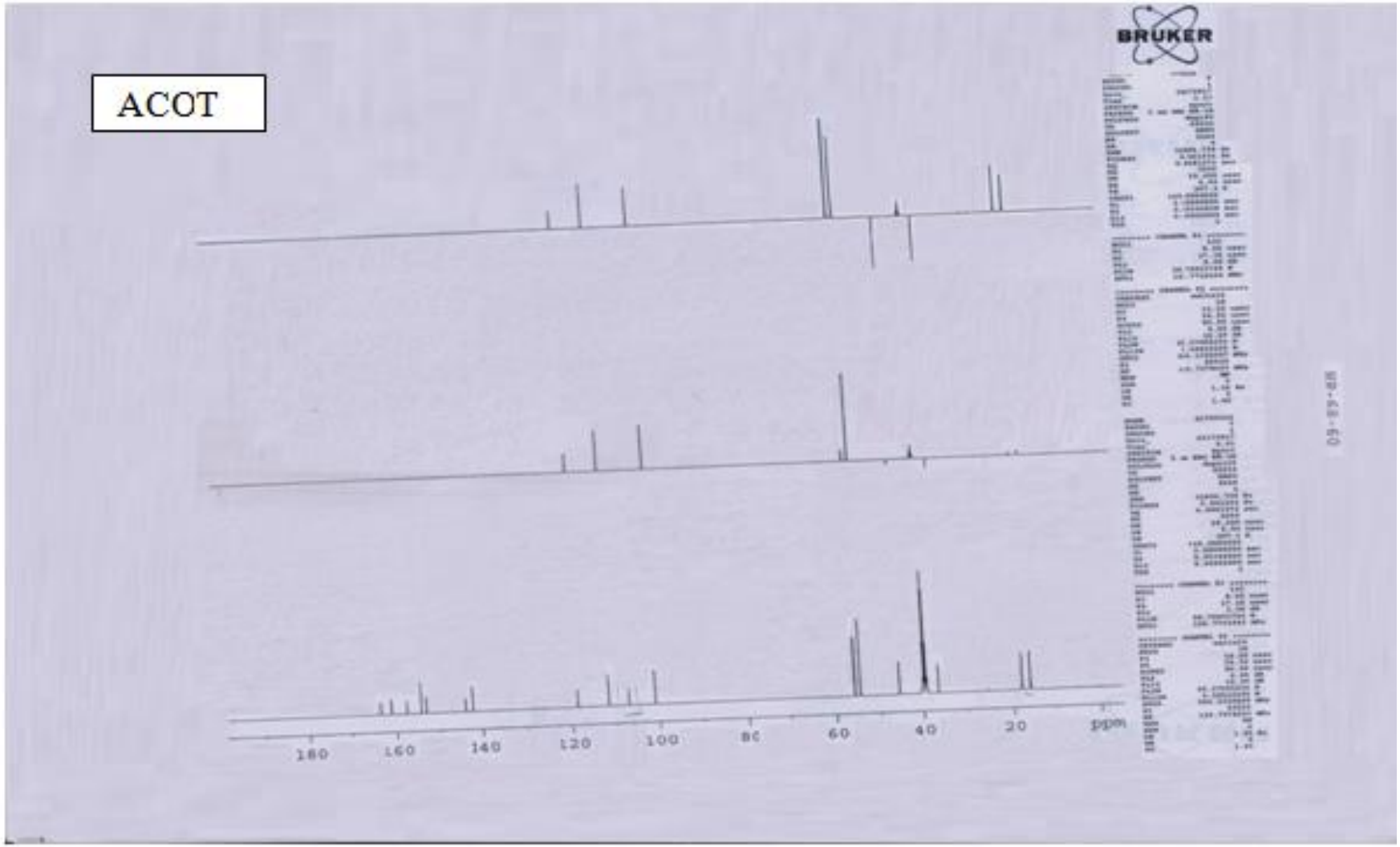


Fig. 4. 30 - DEPT spectra of ACOT

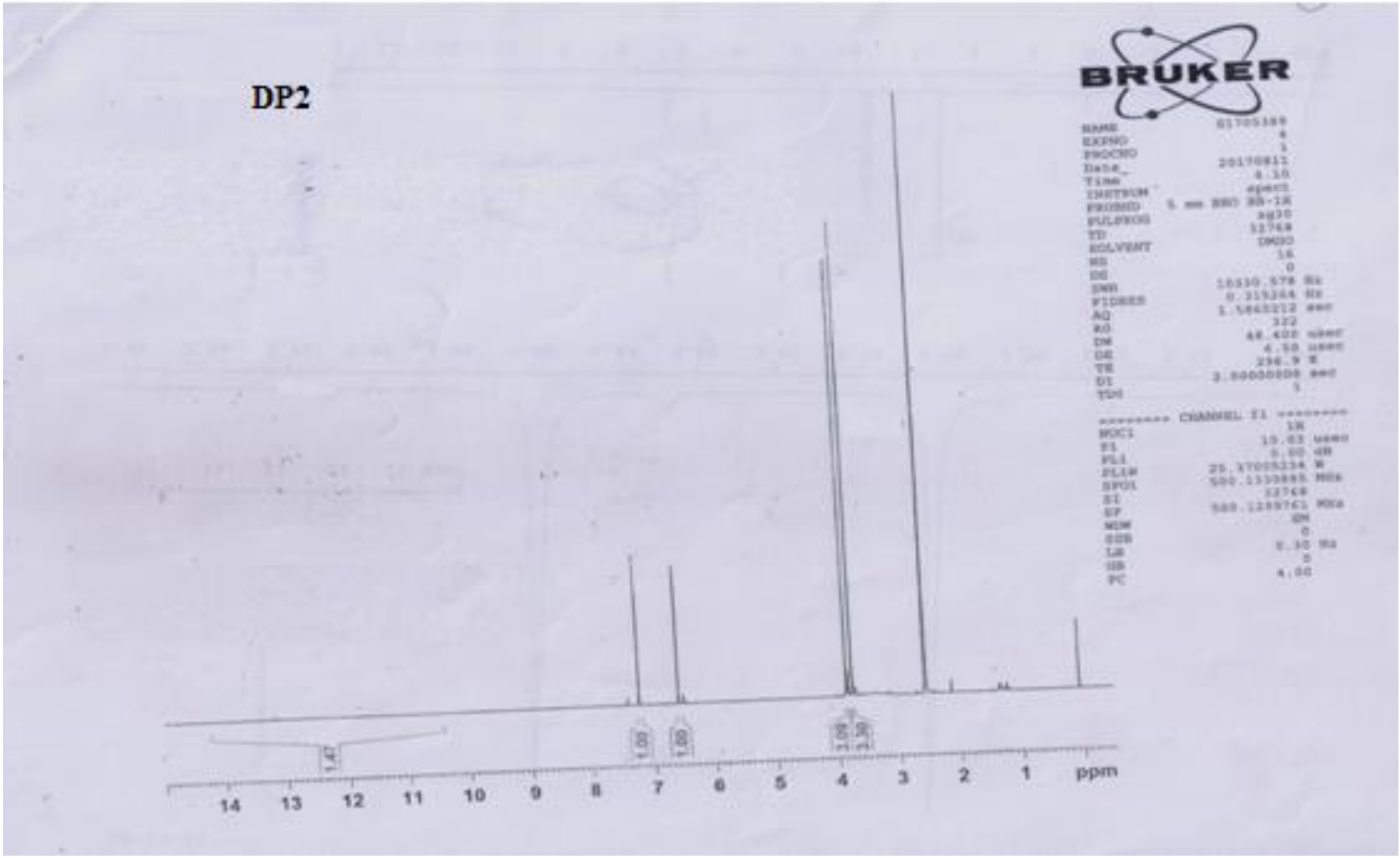


Fig. 4. 31 - ^{1}H NMR spectra of DP2

Fig. 4. 32 - ^{13}C NMR spectra of DP2

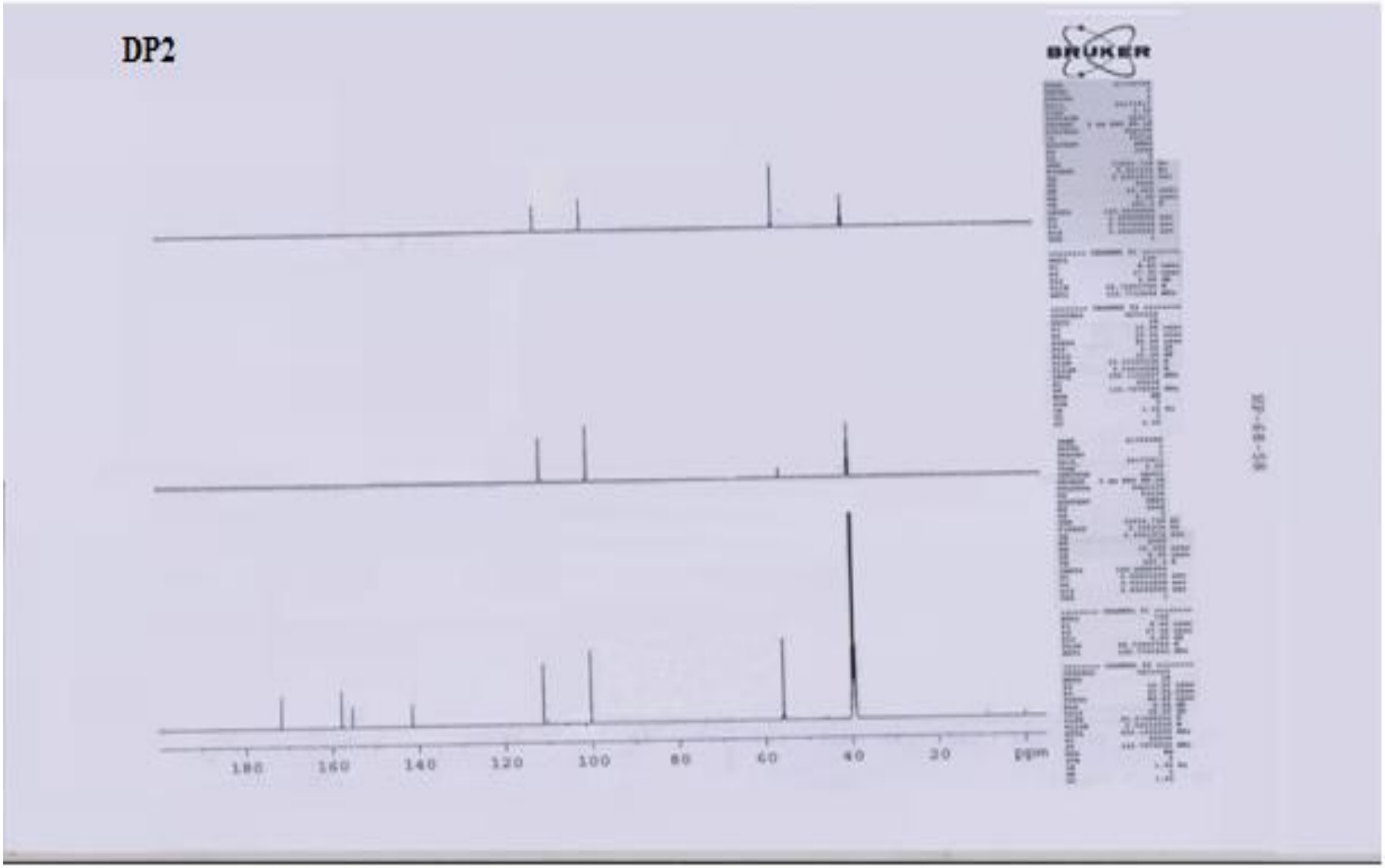


Fig. 4. 33 - DEPT spectra of DP2

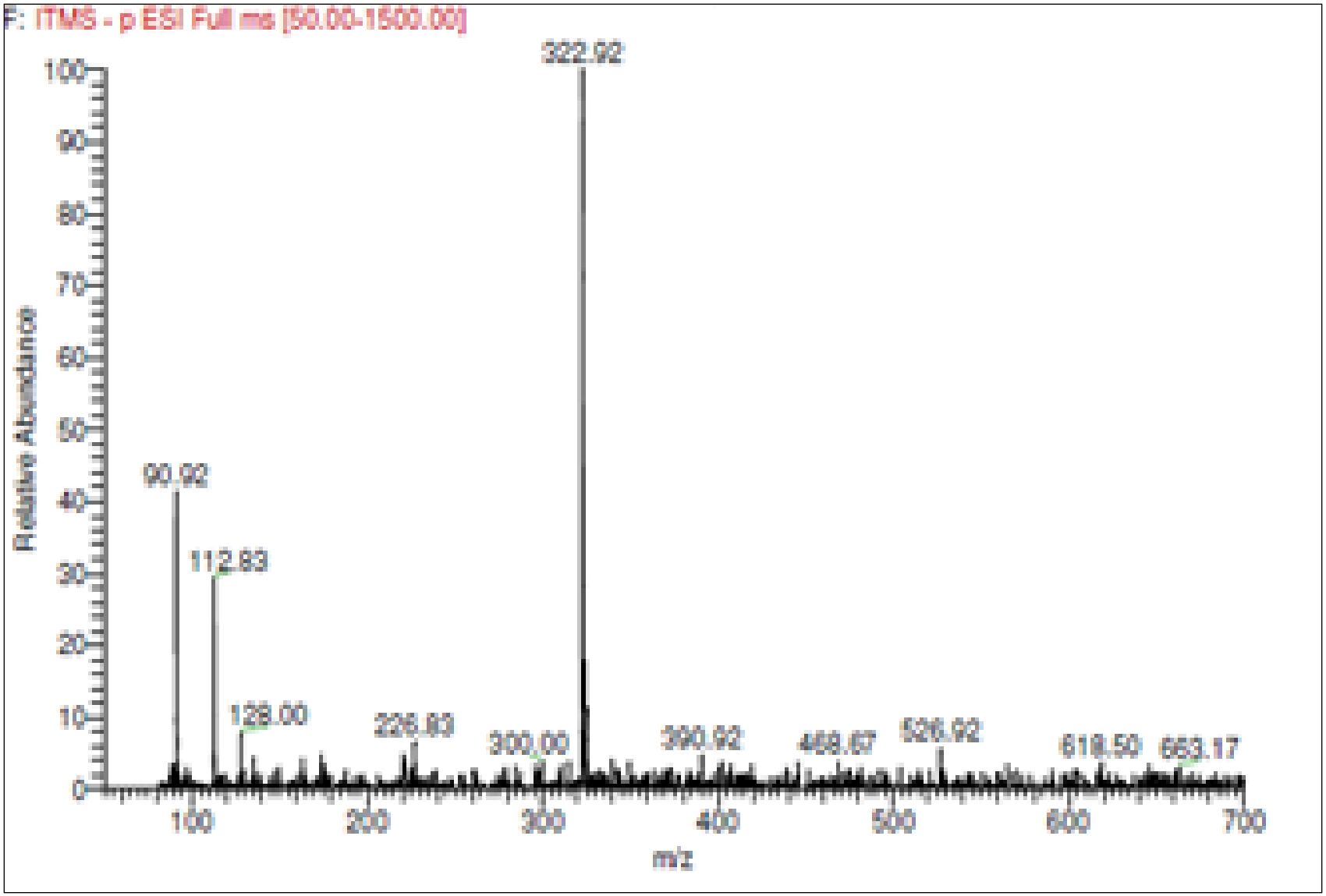


Fig. 4. 34- ESI-MS spectra of DP3

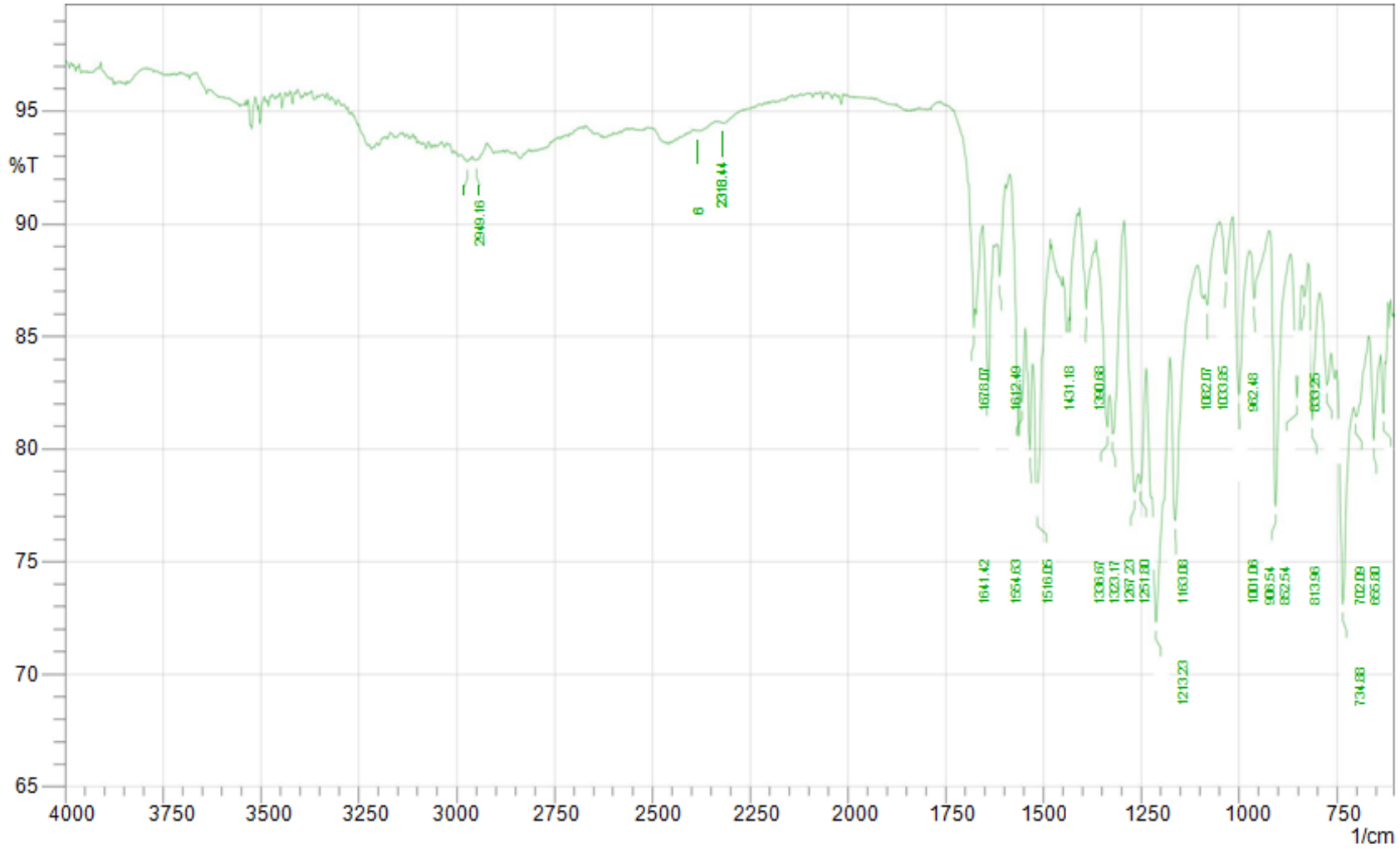


Fig. 4. 35 - I.R. Spectra of DP3

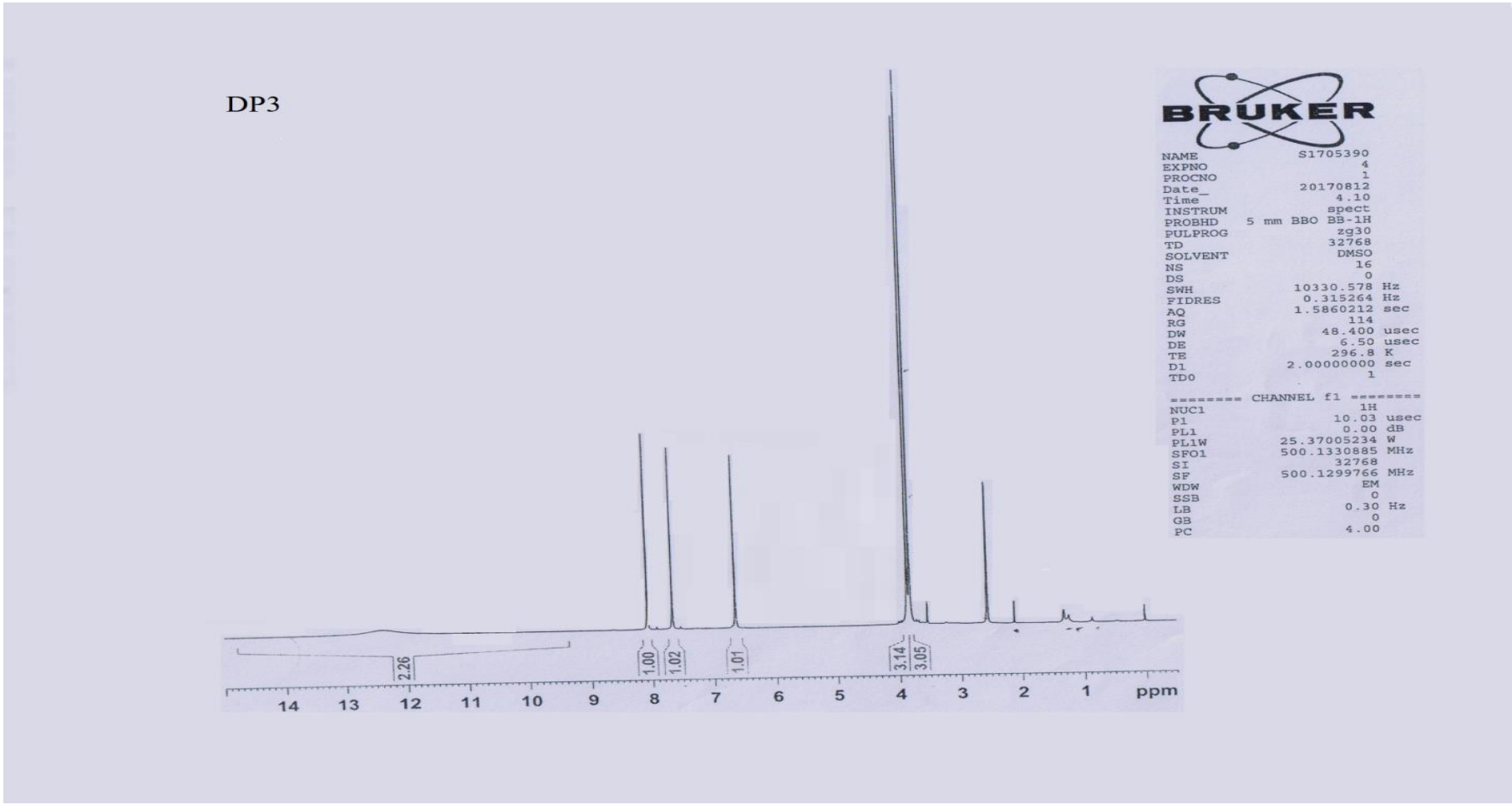


Fig. 4. 36- ^{1}H NMR spectra of DP3

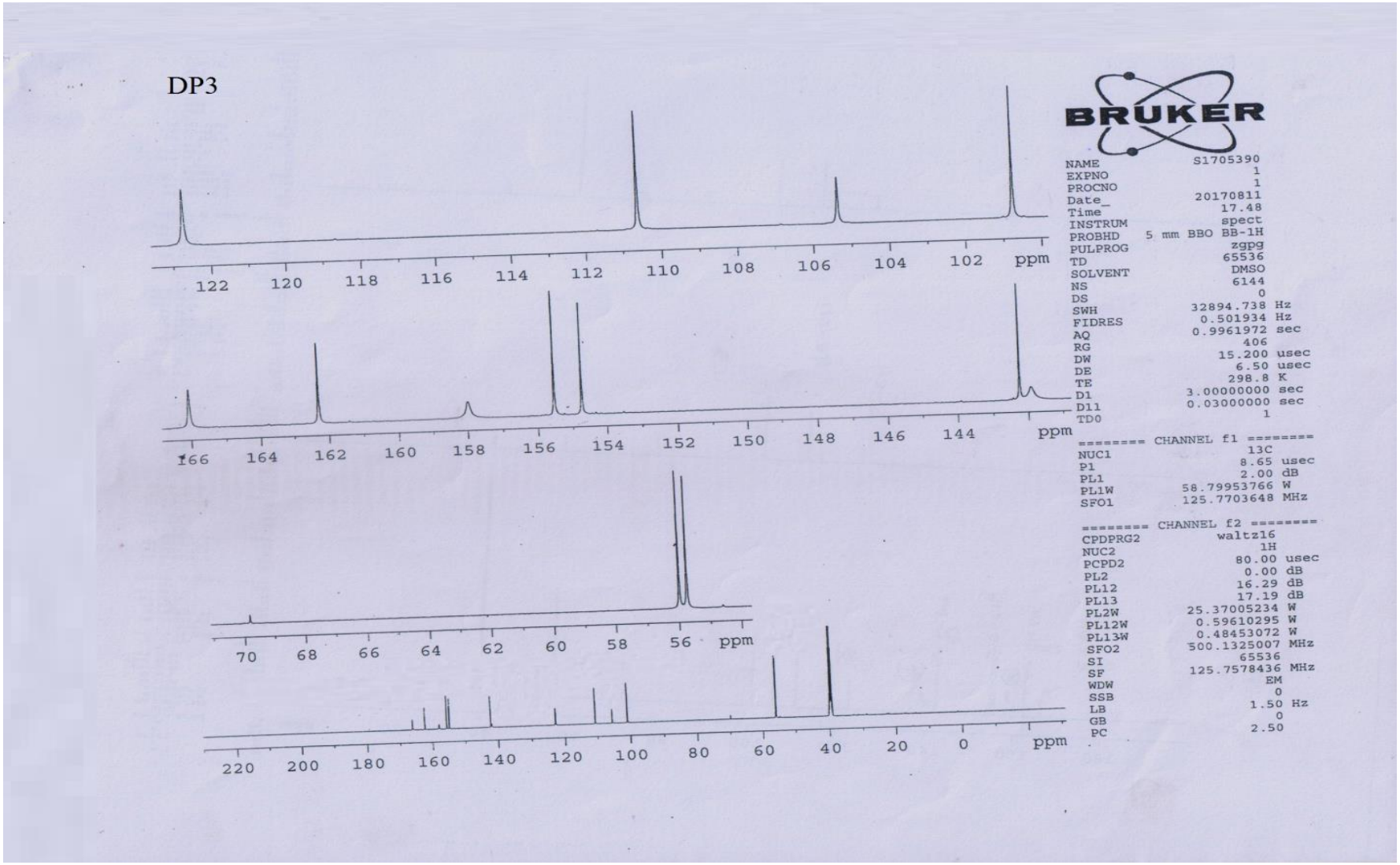


Fig. 4. 37 -^{13}C NMR spectra of DP3

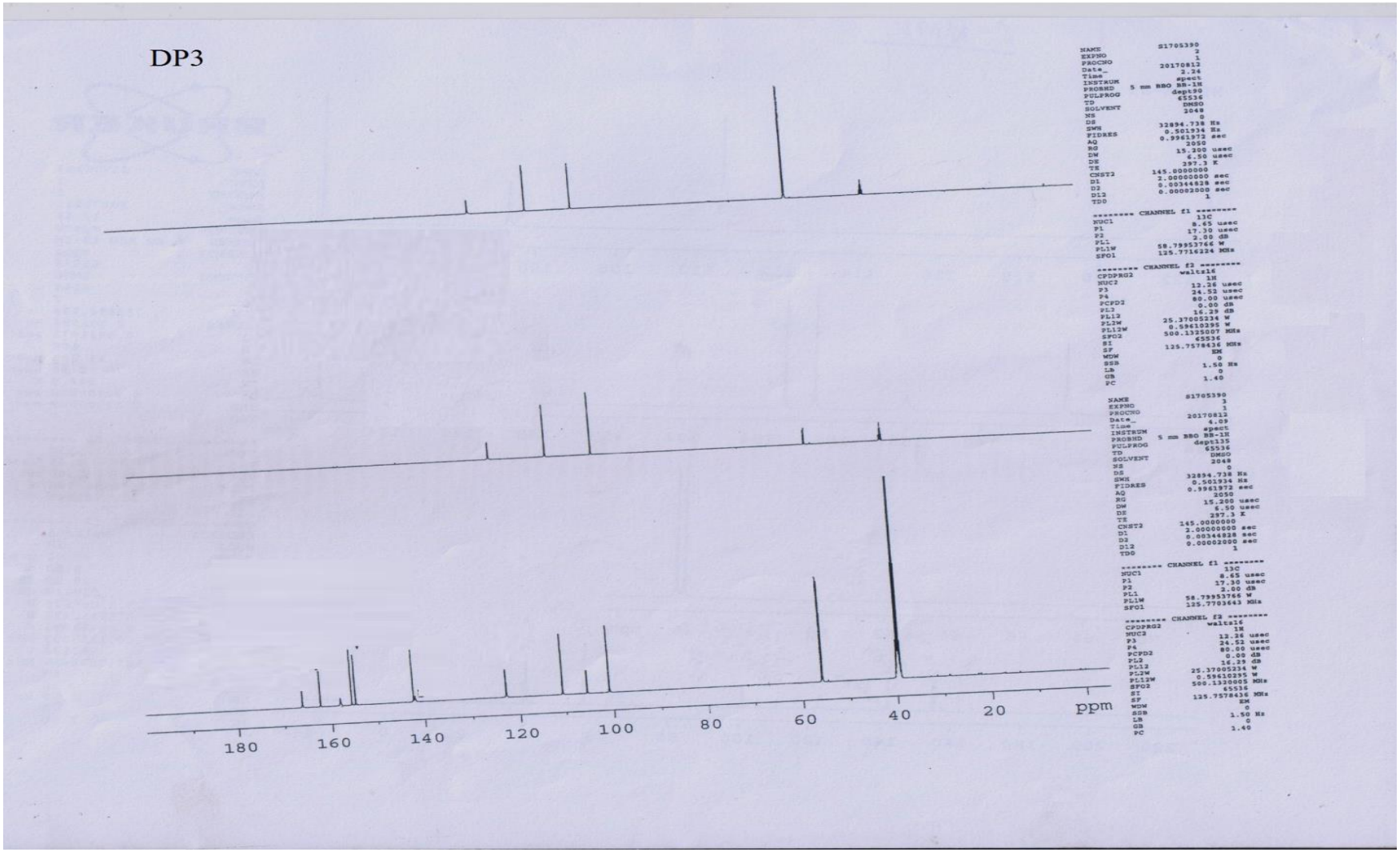


Fig. 4. 38 - DEPT spectra of DP3

4.6. SECTION - C

IMPURITY PROFILING AND DEGRADATION STUDY OF ACOTIAMIDE HCL TRIHYDRATE

4.6.1. EXPERIMENTAL

4.6.1.1. Chemicals and reagents

Chemicals and reagents used in the present section as same as those mentioned in 4.4.1.1.

4.6.1.2. Equipments and Chromatographic conditions

The equipments and chromatographic conditions used in impurity profiling and degradation study are same as those in mentioned in section 4.4.1.3.

For LC-MS analysis, ACOT degradation samples were analysed in same chromatographic conditions as mentioned in section 4.4.1.3.The m/z values were determined in both positive and negative ESI mode. On the basis of molecular weight, structures of DPs were proposed and degradation pathway was postulated.

4.6.1.3. Preparation of stock, sample and buffer solutions

Stock, sample and buffer solutions were prepared in the same was as mentioned in section 4.4.1.5.

4.6.2. RESULTS and DISCUSSION

4.6.2.1. LC-PDA Study

Three degradation products are formed in forced degradation studies of ACOT.
Summary of forced degradation is given in Table 4.15.

Table 4. 15 – Summary of forced degradation study of ACOT analysed by LC-PDA

Stressor	**Conditions**	**RT of degradation products**	**%Degradation (API)**	**% Degradation (Formulation)**
Acidic	1 M HCl 100℃ for 3 hrs	5.361(DP3)	0.92%	0.71%
Alkaline	0.5 M NaOH 100ºC for 3 hrs	3.594 (DP1) 4.079 (DP2) 5.129 (DP3)	15.7%	14.2%
Neutral hydrolysis	100ºc for 6 hrs	--	--	--
Oxidation	6% H_2O_2 RT for 48 hrs	--	--	--
Thermal	Dry at 80℃ for 11 days	--	--	--
Photolytic	5382 Lux and 144 $UVcm^{-2}$ for 11 days	--	--	--

4.6.2.2. LC-MS study and characterization of DPs

ACOT (m/z 451)

Spectral characterization of ACOT by MS/MS and proposed fragmentation pathway is explained in section 4.5.2.1.1.

DP1 (m/z 271)

DP1 is eluted at retention time of 3.5 min. ESI-MS/MS (Fig. 4.39) spectrum of DP1 showed protonated molecular ions at m/z 271. DP1 shows product ions at m/z of 229 by loss of isopropyl group. Further fragments are formed at m/z of 170 and m/z 144 by loss of isopropyl amine and ethyl group. DP1 is formed both in acidic and alkaline condition. DP1 may be formed from ACOT by hydrolysis of amide bond adjacent to thiazole ring. Proposed fragmentation pathway of DP1 is shown in Fig 4.40.

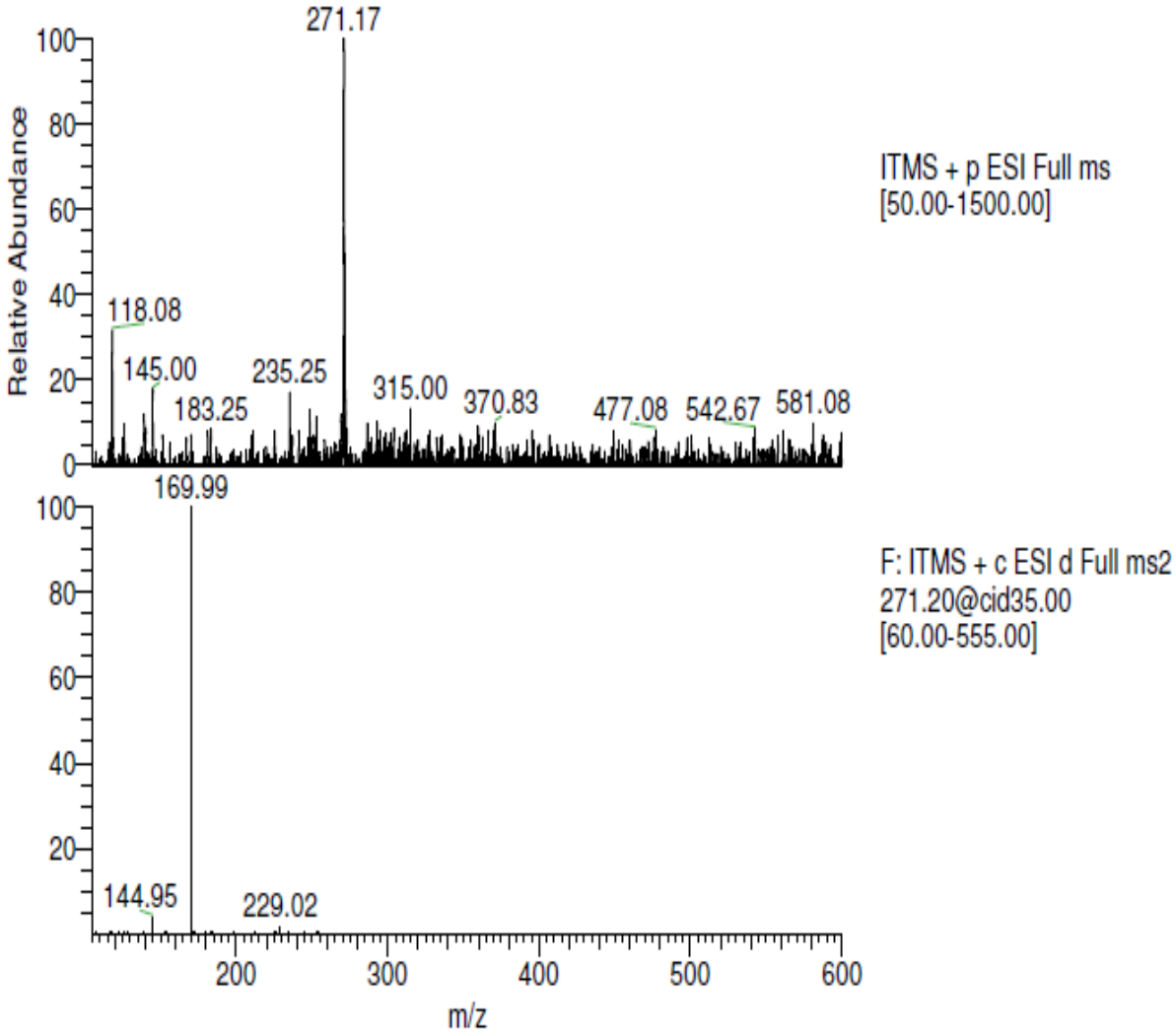


Fig. 4. 39 - ESI-MS/MS spectra of DP1

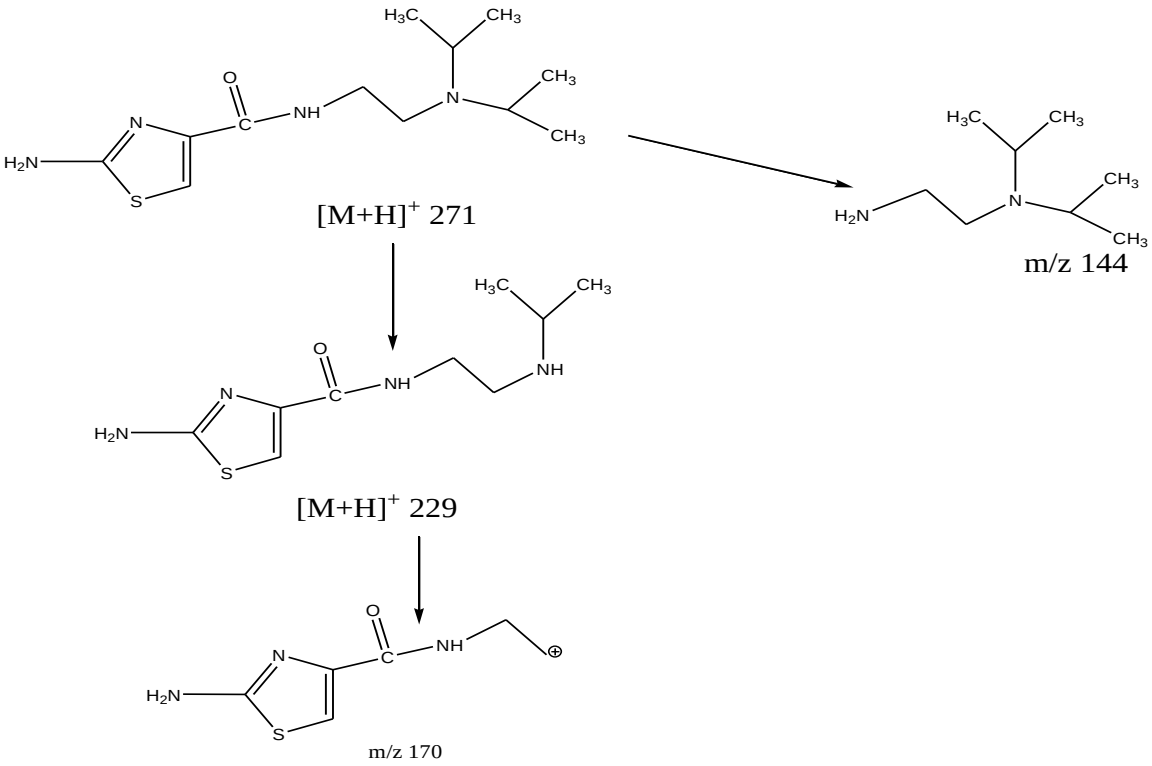


Fig. 4. 40 – Fragmentation pathway of DP1

DP2 (m/z 197)

Mass spectrum interpretation of DP2 is mentioned in section 4.5.2.1.2 and ESI-MS spectra of DP2 is provided in Fig. 4.26.

DP3 (m/z 323)

Mass spectrum interpretation of DP3 is mentioned in section 4.5.2.1.3 and an ESI-MS spectrum of DP3 is provided in Fig.4.34.

4.6.2.3. Degradation pathway of ACOT and mechanism of formation of degradation products

ACOT contains 3 degradation products. DP1 is formed in acidic and alkaline condition. Out of 3 degradation products, degradation products DP2 and DP3 were isolated by preparative HPLC and were characterized by spectral techniques.

Degradation pathway of DP1 and DP2 is shown in Fig 4.41. Alkaline hydrolysis takes place between 10 and 12 position of amide, there is nucleophilic attack of hydroxide ion forming tetrahedral intermediate. In the next step, tetrahedral intermediate with

the help of aqueous solvent dissociates to DP1 and DP2. Degradation pathway of DP3 is shown in Fig.4.42. Alkaline hydrolysis takes place between 18 and 20 position of amide, there is nucleophilic attack of hydroxide ion forming tetrahedral intermediate. In the next step, tetrahedral intermediate with the help of aqueous solvent dissociates to DP3. Chemical structures of ACOT and degradation products are shown in Table 4.16.

Reported method is based on development of stability indicating method using UPLC-Q-TOF-MS using HSS cyano column (100 X 2.1 mm, 1.8 µm), mobile phase: 0.1 % formic acid and acetonitrile in gradient mode. Degradation products are formed in acidic, alkaline, oxidative and photolytic conditions. Comparison of reported method and developed method is shown in Table 4.17.

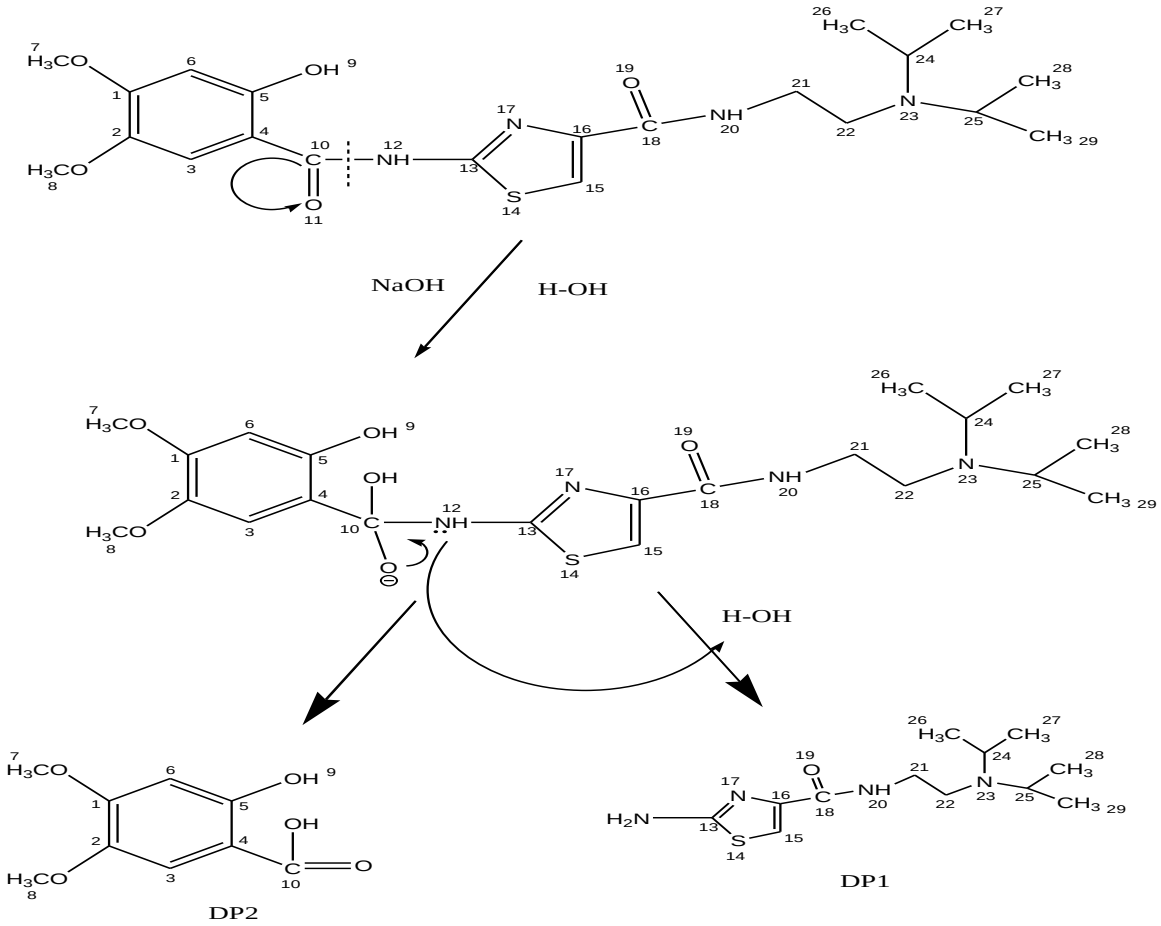


Fig. 4. 41- Degradation pathway of DP1 and DP2

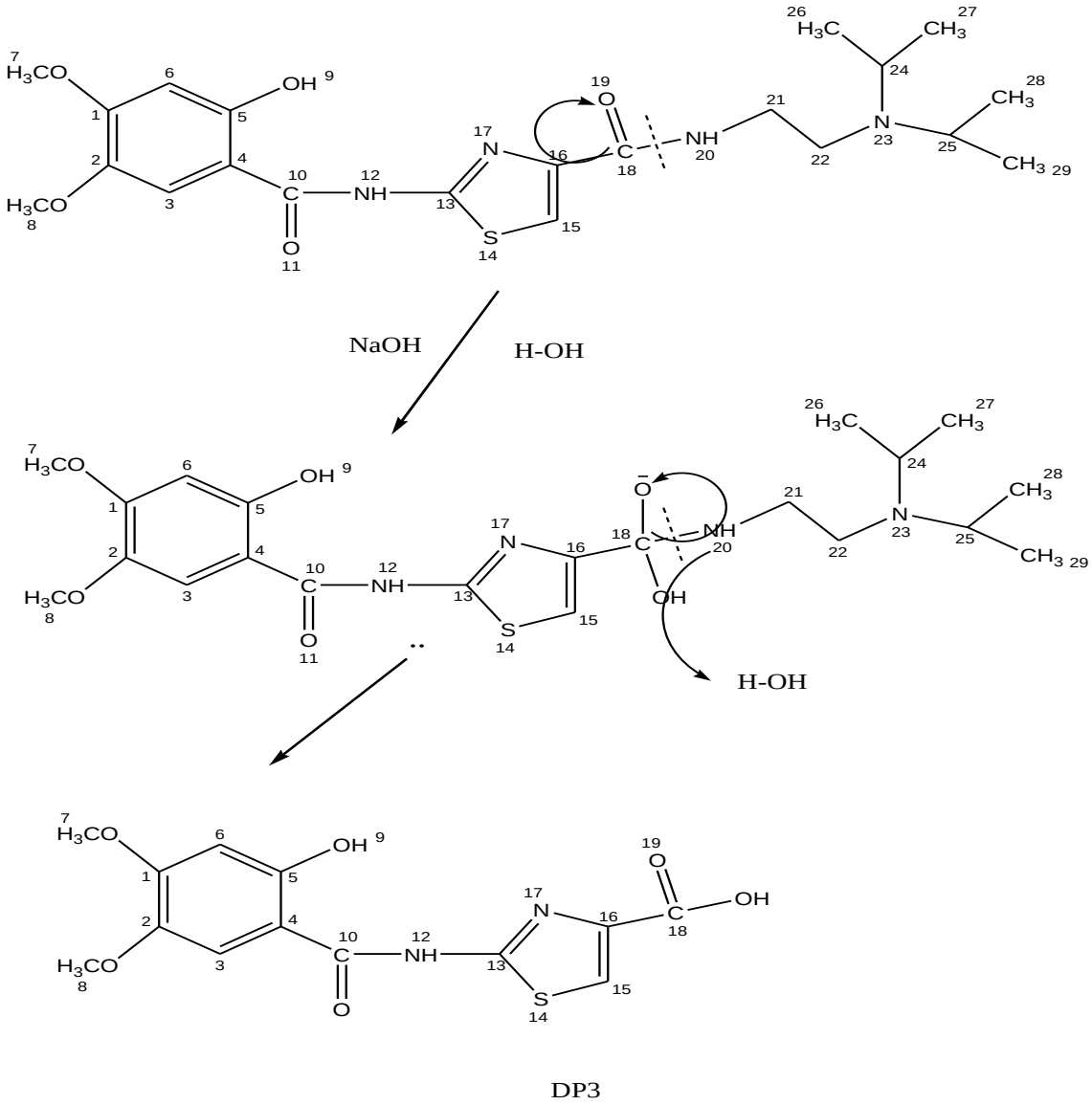
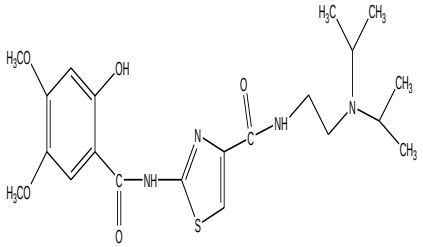
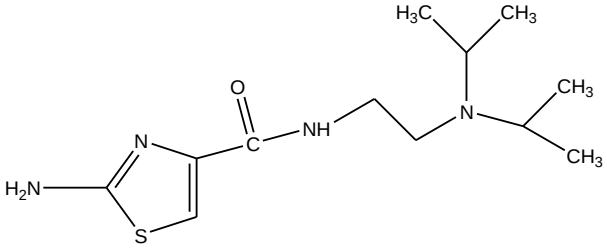
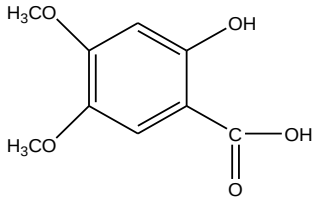


Fig. 4. 42- Degradation pathway of DP3

Table 4. 16 – Chemical structures of ACOT and degradation products

Analyte	**Structure**	**Molecular Formula** **Molecular Weight** **Fragments(m/z)**	**Degradation Route**	**Rt (LC-PDA)**
ACOT		$C_{21}H_{30}N_4O_5S$ 450.19		9.3 min
DP1	 N-(2-(diisopropylamino)ethyl)-2-aminothiazole-4-carboxamide	$C_{12}H_{22}N_4OS$ 270.15 Fragments (229, 169, 145)	Alkaline	3.594 min
DP2	 2-hydroxy-4,5-dimethoxybenzoic acid	$C_9H_{10}O_5$ 198.05	Alkaline	4.079 min

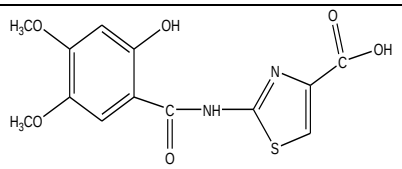
DP3	 2-[(2-hydroxy-4,5-dimethoxybenzoyl)amino]-1,3-thiazole-4-carboxylic acid.	$C_{13}H_{12}N_2O_6S$ 324.04	Alkaline	5.129 min

Table 4. 17 - Comparison of reported method and developed HPLC method

	Methodology	**Degradation conditions**	**% degradation observed**	**Other information**
Literature Ref No. 21	HSS cyano column (100 X 2.1 mm, 1.8 µm), mobile phase : 0.1 % formic acid and acetonitrile in gradient mode, Flow rate : 0.25 mL/min UPLC-ESI-Q-TOFMS/MS	1M HCl 60ºc for 30 hrs	4.4.% (3 DPs)	1 process related impurity
		1M NaOH 60ºc for 30 hrs	9.6% (5 DPs)	
		0.3% H_2O_2 RT 30min	21.76%(5 DPs)	
		Photolytic (1.2 million Lux h and UV light 400 Wh/m2) Solid	No degradation	
		Photolytic (1.2 million Lux h and UV light 400 Wh/m2) Solution	20.9 % (3 DPs)	
Developed HPLC method	RP-HPLC method – C 8 column Mobile phase – 0.1% TEA in 0.2 %	1M HCl 100ºC for 3 hrs	0.92% (1 DP)	DP2 and DP3 isolated and characterized by IR, NMR and Mass
		0.5 M NaOH 100ºC for 3 hrs	15.7 % (3 DPs)	

	formic acid : acetonitrile (70 : 30) Column Oven 40ºC			
		6% H_2O_2 RT 48 hrs	No degradation	
		Photolytic (1.2 million Lux h and UV light 400 Wh/m2) Solid	No degradation	
		Photolytic (1.2 million Lux h and UV light 400 Wh/m2 Solution	No degradation	

4.7. CONCLUSION

Stability indicating method was developed for determination of Acotiamide by HPLC. Significant degradation was observed in alkaline condition and slight degradation was observed in acidic condition. The method developed was validated as per guidelines by ICH. Degradation products were identified by LC-MS. Two degradation products in alkaline condition were isolated and characterized by mass, NMR and IR techniques. The degradation pathway was postulated. The developed method is simple, accurate and sensitive which are applicable for determination of Acotiamide.

4.8. REFERENCES

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