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Dear Editor,

Liquid chromatography/electrospray ionization tandem mass spectrometric study of milnacipran and its stressed degradation products

Stability testing guidelines Q1A (R2) developed by International Conference on Harmonization (ICH) experts require validated stability indicating analytical procedures which are used for stressed samples. Stress testing of drugs facilitates identification of degradation products, degradation pathways and intrinsic stability of drugs. Stress testing includes the effect of temperature, humidity, photolysis, oxidation, and effect of pH on the drug.

Milnacipran (MIL) is a mixed serotonin and nor-epinephrine reuptake inhibitor which is used for the treatment of fibromyalgia. It was approved by the Food and Drug Administration (FDA) in the United States in 2009.[1] Inhibition of both neurotransmitters by MIL leads to synergistic action for treatment of fibromyalgia syndrome and depression.[2] Chemically, MIL is a 2-(aminomethyl)-*N*,*N*-diethyl-1-phenylcyclopropane carboxamide. Few analytical methods have been reported for determination of MIL in biological samples and in pharmaceutical formulations.[3–6] Dias *et al*. have reported a study on the development of a comparative stability indicating high-performance liquid chromatography (HPLC) and second-order derivative UV spectroscopic methods to assay MIL hydrochloride in capsules.[7] Recently, a stability-indicating ultra-performance liquid chromatography (UPLC) method for MIL has been reported.[8] However, no information exists in the literature on the degradation behavior and stability indicating an LC/MS method of MIL. Hence, the present study focuses on the identification and structural characterization of the degradation products of MIL by using LC/MS/MS and accurate mass measurements.

Pure MIL was obtained as a gratis sample from Sebondscience Labs (Hyderabad, India). Acetonitrile, ammonium acetate, methanol, sodium hydroxide, hydrochloric acid and hydrogen peroxide were purchased from Merck (Darmstadt, Germany). All reagents used were of analytical grade and the acetonitrile was HPLC grade. Water was purified by a Milli-Q® system (Progard 2; Millipore, Milford, MA, USA).

The HPLC analysis was performed on an Agilent 1200 series HPLC instrument (Agilent Technologies, USA) equipped with a quaternary pump (G13311A), a de-gasser (G1322A), a diode-array detector (G1315D), an autosampler (G1329A, USA) and a column compartment (G1316A). For LC/MS analysis, an Agilent 1200 series HPLC instrument (Agilent Technologies, USA) was coupled to a quadrupole time-of-flight mass spectrometer (Q-TOF LC/MS 6510 series classic G6510A, Agilent Technologies, USA) equipped with an electrospray ionization (ESI) source. The data acquisition was under the control of Mass Hunter workstation software. The ESI source conditions were optimized as follows: fragmentor voltage, 80 V; capillary voltage, 3000–3500 V; skimmer, 60 V; nitrogen was used as drying (300°C; 9 L/min) and nebulizing (45 psi) gas.[9] For full scan MS mode, the mass range was set at *m/z* 100–3000. For collision-induced dissociation (CID) experiments keeping MS^1 static, the precursor ion of interest was selected using the quadrupole analyzer and the product ions were analyzed using a TOF analyzer. Ultra-high purity nitrogen was used as collision gas. All the spectra were recorded under identical experimental conditions and are an average of 20–25 scans. A splitter was placed before the ESI source, allowing entry of only 35% of the eluent.

A water bath equipped with a temperature controller was used to carry out degradation studies. A controlled temperature dry air oven (Mack pharmatech Pvt. Ltd., Mumbai, India) was used for solid-state thermal stress studies. A photostability chamber (Sanyo, Leicestershire, UK) was used for the photodegradation study which consists of both a UV and a fluorescent lamp. A calibrated lux meter and UV meter

Table 1. Parameters of linear regression equation

Parameter	Value
Calibration range (ng mL^{-1})	10–60
Correlation coefficient (r^2)	0.9981
Slope	12034
Intercept	31043
SD of slope	261.50
SD of intercept	10184

Table 2. Intra- and inter day precision data of MIL

	Intra-day precision	Inter-day precision
Concentration (ng mL^{-1})	Measured concentration (ng mL^{-1}) SD; RSD (%)	Measured concentration (ng mL^{-1}) SD; RSD (%)
30	30.70 ± 0.0051; 0.016	30.85 ± 0.0615; 0.19
50	49.73 ± 0.0498; 1.00	49.43 ± 0.042; 0.08
60	60.37 ± 0.4971; 0.82	59.35 ± 0.059; 0.10

Table 3. Recovery data of MIL

Spiked concentration (ng mL^{-1})	Calculated spiked concentration (ng mL^{-1}) SD; RSD (%)	Recovery (%)
25	24.736 ± 0.0270; 0.109	98.946
30	30.199 ± 0.0504; 0.166	100.66
35	34.959 ± 0.0451; 0.129	99.88

were used to measure energy. All pH measurements were carried out on a pH meter (Metrohm Schweiz AG, 780 pH meter, Germany) with Epson printer Lx-300t and weighing was done on a Sartorius balance (CD 225 D, 22308105 Germany).

Stress degradation studies of MIL were carried out under hydrolytic (acid, base and neutral), oxidative, dry heat and photolytic stress conditions as mentioned in ICH Q1A (R2) (2003)[10] and ICH Q1B (1996)[11] guidelines. Acidic, basic and neutral hydrolysis was carried out by refluxing MIL with 1 N HCl, 0.1 N NaOH and water for 5 h, 15 h and 15 h, respectively. The oxidative stress degradation study was carried out with 15% H_2O_2 for 15 h at room temperature. MIL was spread over a petri dish and kept at 60°C for 24 h for the thermal degradation study. Similarly, the solid-state photolytic study was carried out by exposing MIL to 1.2×10^6 lux h of fluorescent light and 200 W h m^{-2} UV light in a photostability chamber. All degradation studies were carried out with a drug concentration of 1 mg mL^{-1}. All the stressed samples were withdrawn at suitable time intervals and diluted with diluents (methanol/acetonitrile, 1:1). All solutions were filtered through a 0.22 µm pore size nylon 66 membrane filter before HPLC and LC/MS analysis.

The separation of MIL and its degradation products was achieved on a Waters symmetry C-18 column (150 × 4.6 mm, 5 µm) using a mobile phase consisting of solvent A (0.1% trifluoroacetic acid) and solvent B (acetonitrile). The flow rate of the mobile phase was 0.5 mL min^{-1}, the detection wavelength

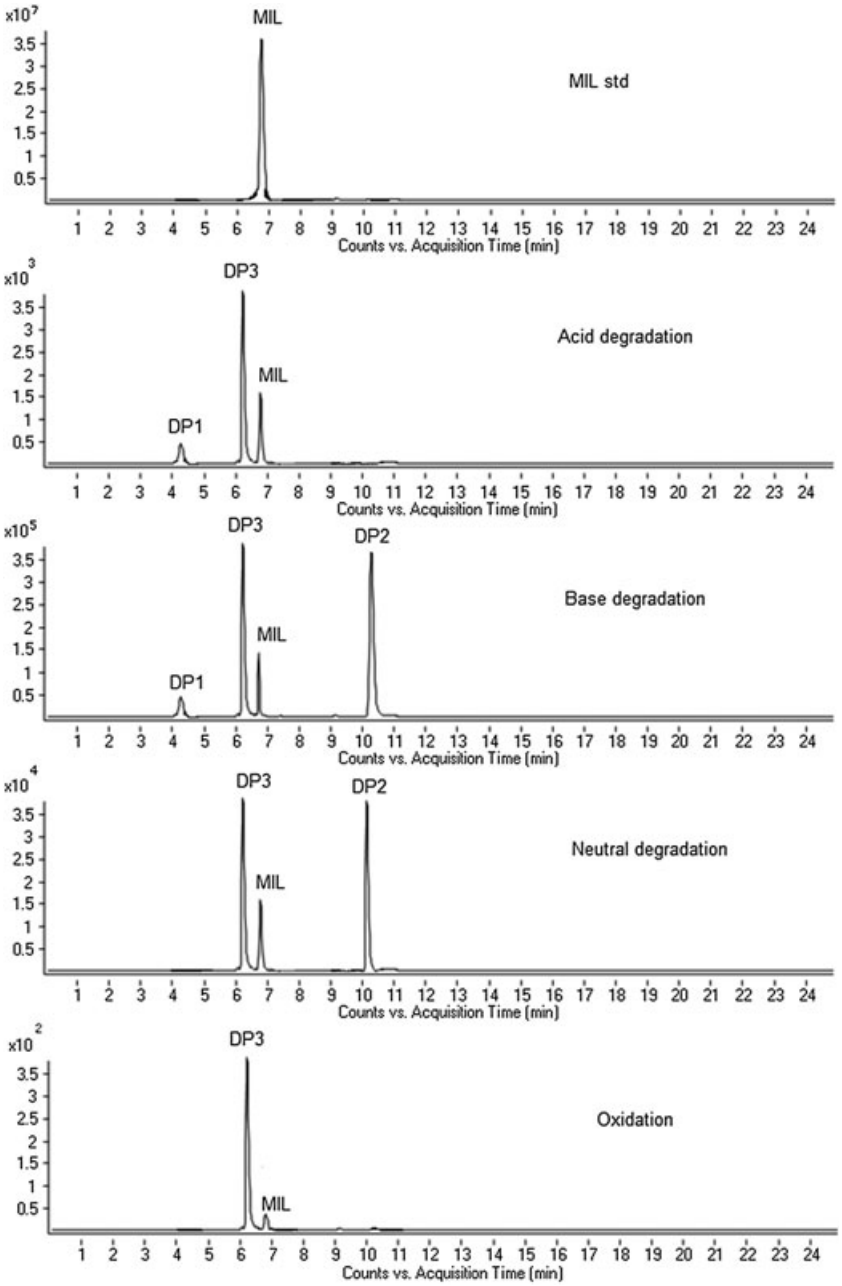

Figure 1. LC/ESI-MS total ion chromatogram (TIC) of MIL and its degradation products formed under hydrolytic (acidic, basic and neutral) and oxidative stress degradation conditions.

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was at 220 nm, the column oven temperature was set at 25°C and the injection volume was 10 µL. The gradient elution method was (T_{min}/% solution B): $_0$/20, $_5$/40, $_{10}$/60, $_{15}$/60, $_{25}$/20.

The stability-indicating LC/MS assay method was validated with respect to linearity, precision (inter-day and intra-day), accuracy and specificity as summarized in the ICH guidelines Q2 (R1).[12] To establish linearity and range, a stock solution containing 1 mg mL^{-1} MIL in diluent was diluted to yield solutions in the concentration range of 10–60 ng mL^{-1}. The linearity test solutions were prepared and analyzed in triplicate. The correlation coefficient value obtained from the linear regression graph was 0.9981. The relative standard deviation (% RSD) for each linearity test concentration was <0.18%. The linearity data are given in Table 1. The intra- and inter-day precision were determined at the concentrations of 30, 50 and 60 ng mL^{-1} on the same day ($n = 3$) and on consecutive days ($n = 3$). The %RSD values for intra-day and inter-day precision studies were <1.0% and <2%, respectively (Table 2) which confirms that the method is sufficiently precise. The recoveries of the added drug were obtained from the difference between peak areas of fortified and unfortified degraded samples. The recovery of MIL in the presence of degradation products ranged from 98 to 101% (Table 3). The specificity of the method was evaluated by determining peak purity for MIL in a mixture of stressed samples using the PDA detector. Peak purity was determined by purity angle and purity threshold.

Figure 1 shows the LC/ESI-MS total ion chromatogram (TIC) of degradation products formed under various stress conditions. The drug degraded under hydrolysis (acidic, basic and neutral) and oxidation conditions, while it was stable under other degradation conditions. Three degradation products (**DP-1** to **DP-3**) were formed.

The positive ion ESI-MS spectrum of MIL shows an abundant $[M+H]^+$ ion at *m/z* 247. Its MS/MS spectrum (Fig. 2(a)) shows product ions at *m/z* 230 (loss of NH_3), 202 (loss of C_2H_4 from *m/z* 230), 159 (loss of C_2H_5N from *m/z* 202), 129 (loss of HCHO from *m/z* 159), 100 (((diethylamino)methylidyne)

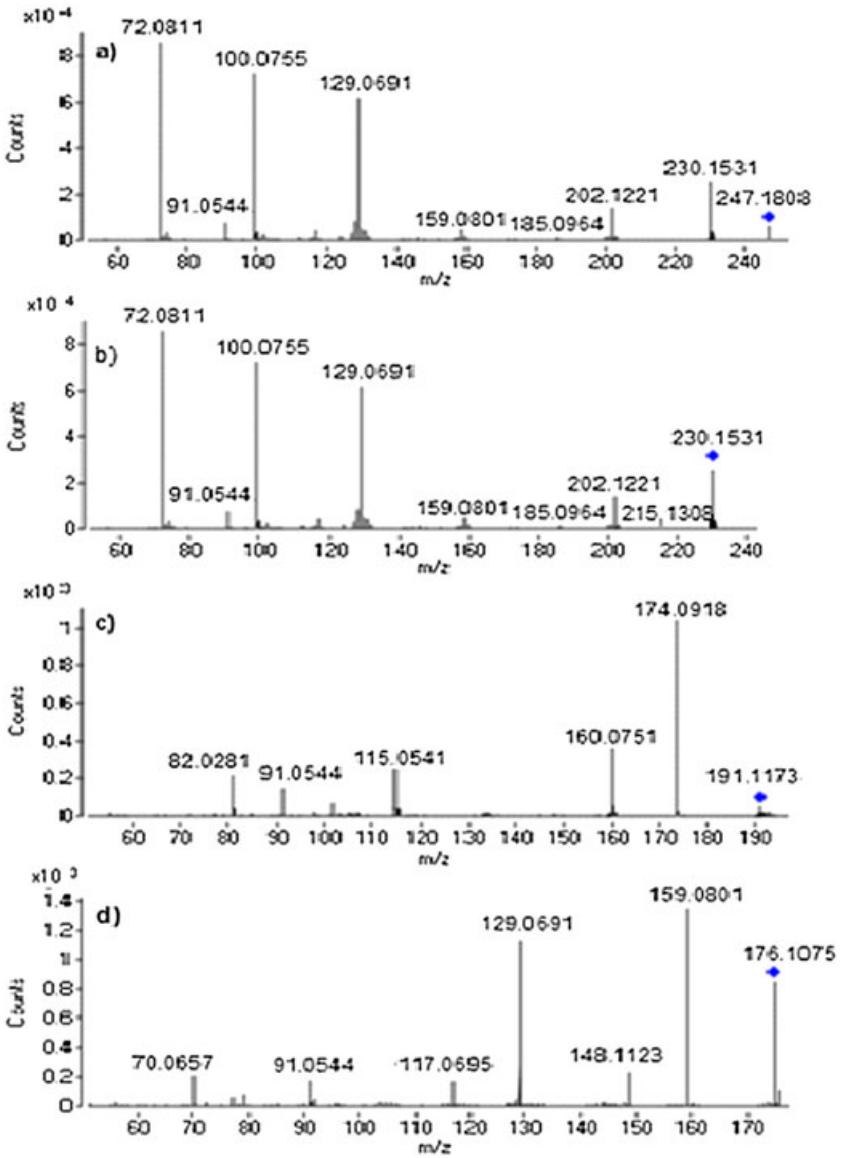


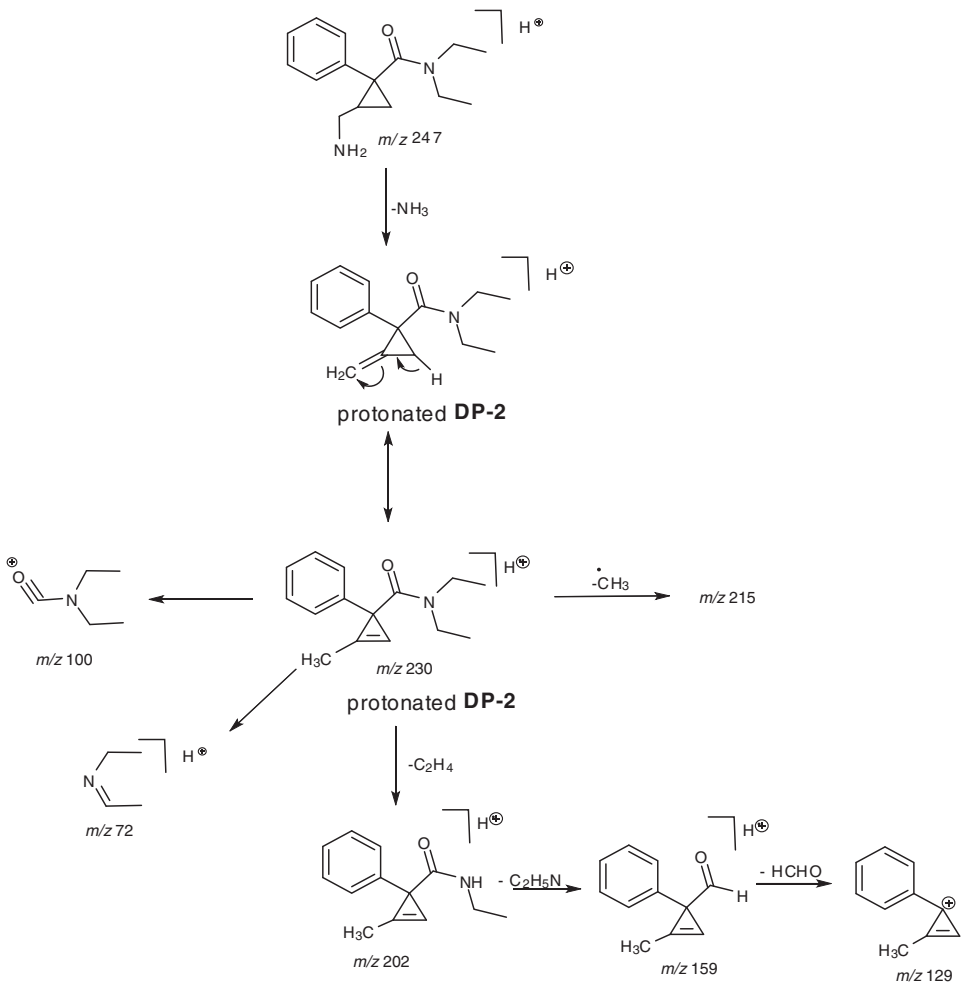
Figure 2. LC/MS/MS spectra of (a) protonated **MIL** (*m/z* 247), (b) protonated **DP-2** (*m/z* 230), (c) protonated **DP-1** (*m/z* 191), and (d) protonated **DP-3** (*m/z* 176) at 18 eV.

oxonium) and 72 (protonated *N*-ethylideneethanamine) (Scheme 1). The high-resolution mass spectrometric (HRMS) data of the product ions are given in Table 4.

The degradation product **DP-1** formed under acidic and basic conditions was eluted at 4.2 min. The LC/HRMS spectrum shows its $[M+H]^+$ ion at *m/z* 191.1173 with an elemental composition of $C_{11}H_{15}N_2O$. Its MS/MS spectrum (Fig. 2(c)) shows product ions at *m/z* 174 (loss of NH_3), 160 (loss of CH_3NH_2), 115 (1-phenylcycloprop-2-en-1-ylium) and 82 (1-carbamoylcycloprop-2-en-1-ylium) (Scheme 2). The formation of *m/z* 82 is indicative of the presence of the cyclopropanecarboxamide group. From the structure of MIL, the observed fragmentation of protonated **DP-1**, supported by accurate mass measurements (Table 4), is found to be highly compatible with the proposed structure, 2-(aminomethyl)-1-phenylcyclopropanecarboxamide.

The degradation product **DP-2** at *m/z* 230.1531 ($[M+H]^+$: $C_{15}H_{20}NO$), formed under basic and neutral stress conditions, was detected at 10.1 min. The HRMS data suggests that it is formed by the loss of NH_3 from the protonated drug. The *m/z* 230 ion probably undergoes a rearrangement through a 1,3-H migration to a stable structure corresponding to protonated **DP-2**, as shown in Scheme 1. This is supported by the LC/MS/MS spectrum of protonated **DP-2** (Fig. 2(b)) which clearly shows a diagnostic low-abundance product ion at *m/z* 215 (loss of CH_3) in addition to other characteristic ions. Based on these data combined with accurate mass measurements, **DP-2** was identified as *N,N*-diethyl-2-methyl-1-phenylcycloprop-2-enecarboxamide.

The degradation product **DP-3** at *m/z* 176.1075 ($[M+H]^+$: $C_{11}H_{14}NO$), formed under hydrolytic (acidic, basic and neutral) and oxidative stress conditions, was eluted at 6.2 min. The HRMS data suggests that **DP-3** was formed by the loss of the *N*-ethylidene ethanamine moiety from the drug. This reaction is probably initiated by protonation of the carbonyl oxygen of the amide, which activates it toward nucleophilic attack by water. The nucleophilic attack assists in the elimination of diethylamine resulting in the formation of a *gem*-diol intermediate and subsequently loss of a water molecule results in the formation of **DP-3** (Scheme 3). A similar mechanism involving a *gem*-diol intermediate appears to be operational under basic and neutral conditions, probably due to usage of a sufficient amount of water during work-up of the reaction mixture. The LC/MS/MS spectrum (Fig. 2(d)) of protonated **DP-3** shows characteristic product ions at *m/z* 159 (loss of NH_3), 148 (loss of CO), 129 (loss of HCHO from *m/z* 159), 117 (loss of CH_3NH_2 from *m/z* 148) and 70 (loss of C_6H_6 from *m/z* 148) (Scheme 4). The formation of the fragment ion at *m/z* 117 points to the presence of a



Scheme 1. Proposed fragmentation mechanism for **MIL** (*m/z* 247) and protonated **DP-2** (*m/z* 230).

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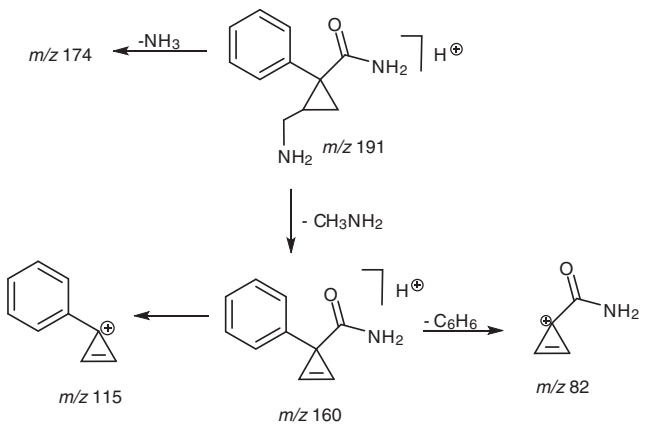
Table 4. HRMS data of product ions of protonated MIL and its degradation products

MIL and degradation products	Rt (min)	Proposed formula	Observed mass (Da)	Calculated mass (Da)	Error (ppm)	Proposed neutral loss
MIL and **DP-2**	6.8 & 10.1	$C_{15}H_{23}N_2O$	247.1808	247.1805	−0.92	-
		$C_{15}H_{20}NO$	230.1531	230.1539	3.36	NH_3
		$C_{14}H_{17}NO$	215.1309	215.1305	−2.11	CH_3
		$C_{13}H_{16}NO$	202.1221	202.1226	2.69	C_2H_4
		$C_{11}H_{11}O$	159.0801	159.0804	0.91	C_2H_5N
		$C_{10}H_9$	129.0691	129.0699	3.32	HCHO
		$C_5H_{10}NO$	100.0755	100.0757	0.79	$C_{10}H_{10}$
		$C_4H_{10}N$	72.0811	72.0808	−0.86	$C_{11}H_{13}NO$
DP-1	4.2	$C_{11}H_{15}N_2O$	191.1173	191.1179	2.12	C_4H_8
		$C_{11}H_{12}NO$	174.0918	174.0913	−2.45	NH_3
		$C_{10}H_{10}NO$	160.0751	160.0757	2.93	CH_3NH_2
		C_9H_7	115.0541	115.0542	0.11	CH_3NO
		C_4H_4NO	82.0281	82.0287	3.31	C_6H_6
DP-3	6.2	$C_{11}H_{14}NO$	176.1075	176.1070	−2.42	C_4H_9N
		$C_{11}H_{11}O$	159.0801	159.0804	0.91	NH_3
		$C_{10}H_{14}N$	148.1123	148.1121	−0.67	CO
		$C_{10}H_9$	129.0691	129.0699	3.61	HCHO
		C_9H_9	117.0695	117.0699	2.11	CH3NH$_2$
		C_4H_8N	70.0657	70.0651	−3.11	C_6H_6

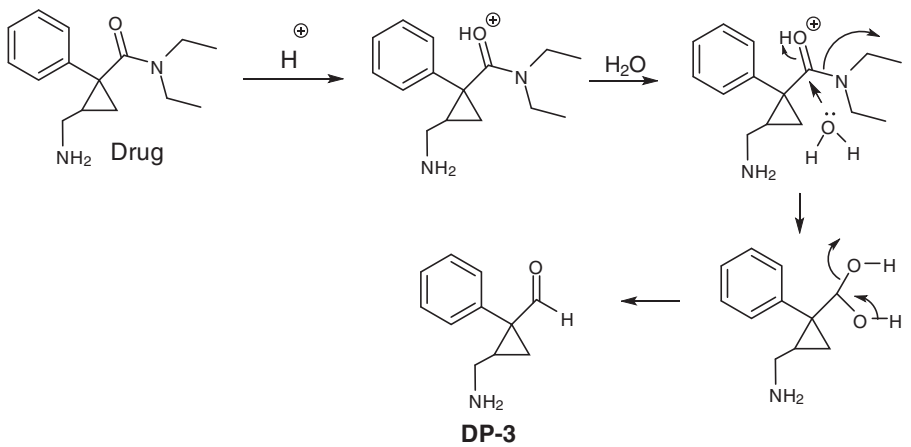
Rt: retention time



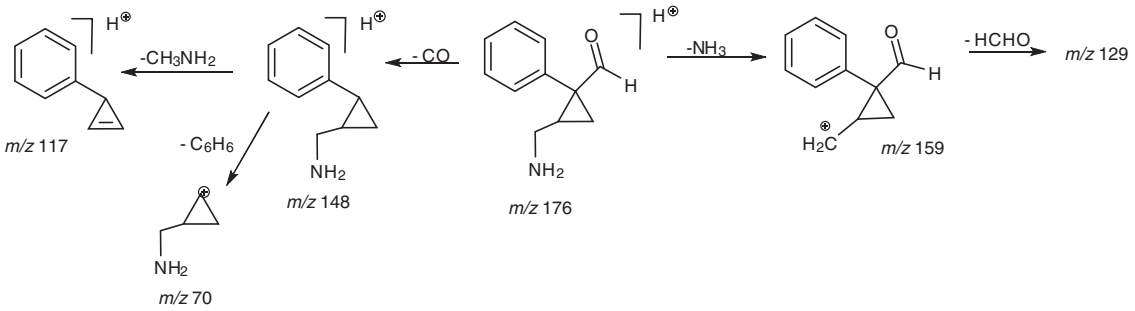
Scheme 2. Proposed fragmentation mechanism for protonated **DP-1** (*m/z* 191).

cyclopropylbenzene moiety in the structure of **DP-3**. Based on MS/MS experiments and accurate mass measurements (Table 4), the structure of **DP-3** can be assigned as 2-(aminomethyl)-1-phenylcycloprop-2-enecarbaldehyde.

In conclusion, stress degradation studies on MIL were carried out according to ICH guidelines, providing information on the degradation behavior of the drug under hydrolysis and oxidation conditions. The liquid chromatography method described here can resolve all the degradation products from milnacipran as well as from each other under various stress conditions. The drug degraded under hydrolysis and oxidation conditions, while it was stable under other degradation conditions. A total of three degradation products were formed and characterized with the help of LC/MS/MS combined with accurate mass measurements.



Scheme 3. Proposed mechanism for the formation of **DP-3** under hydrolysis conditions.



Scheme 4. Proposed fragmentation mechanism for protonated **DP-3** (*m/z* 176).

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