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Characterization and *In-silico* toxicity prediction of degradation products of felbamate

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Abstract

Background: The objective of the work carried out was to assess the toxicity of the degradation products (DPs) for the drug felbamate. Stress studies were performed in the condition specified in the international council of harmonization (ICH) guideline Q1A (R2).

Results: The drug degraded under the alkaline stress conditions to generate two degradation products (DPs). They were separated on a Phenomenex C8 column (250 mm × 4.6 mm, 5 μm); mobile phase composition was 10 mM ammonium formate (pH adjusted to 3.7 with formic acid) and acetonitrile (80:20, v/v); flow rate and wavelength for recording absorbance were 1.0 ml/min and 206 nm, respectively. The structures of the degradation products were characterized by LC–MS/MS analysis.

Conclusion: The drug was prone to hydrolysis in the presence of alkali. It was found to be stable under other stress conditions, viz., acidic, neutral, thermal, photolytic and oxidative. The structures of the impurities were characterized by LC–MS/MS. The drug and the DPs were screened through ADME and toxicity prediction software's like pkCSM, Toxtree and OSIRIS property explorer. Felbamate was flagged for possible hepatotoxicity.

Keywords: Felbamate, Stress studies, *In-silico* toxicity, Mass spectrometry

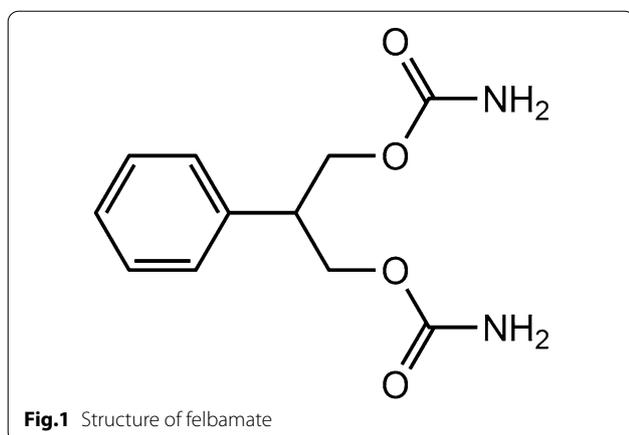
Background

Felbamate is an antiepileptic drug. It is one of the first in its class belonging to the second generation. In adults, it is used in the treatment of partial seizures [1], while in children for generalized seizures due to Lennox-Gas taut syndrome [2, 3].

Felbamate is official in the US Pharmacopeia and National Formulary (USP-NF) [4]. It is also mentioned in Clarke's Analysis of Drugs and Poisons [5]. Chemically felbamate is 2-phenyl-1,3-propanediol (Fig. 1). It is a white lipophilic powder, very slightly soluble in water, slightly soluble in alcohol, but freely soluble in organic solvents [6].

Studies have shown that the antiepileptic activity of felbamate is attributed primarily due to the inhibitory effect on GABA_A receptor. Secondly, it is also known to inhibit the glycine-mediated activation of the NMDA receptor. The GABA_A receptor is known to modulate the chloride channel, whereas the NMDA receptor control sodium and calcium passage. Extracellular magnesium can bind to NMDA receptors blocking the movement of cations across the membrane. These voltage-dependent ion channels control neuronal excitability and membrane stabilization [7, 8]. After administration, felbamate is metabolized in the liver by cytochrome P-450, followed by glucuronidation. Approximately half of the absorbed felbamate is excreted in the urine. There are three major metabolites of felbamate: 2-(4-hydroxyphenol)-1,3-propanediol dicarbamate, 3-carbamoyloxy-2-phenylpropanoic acid and 2-hydroxy-2-phenylpropanoic acid. The minor metabolite is 2-hydroxy-2-phenyl-1,3-propanediol

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monocarbamate. There is no reported therapeutic activity associated with any of these metabolites [9].

There are a few bioanalytical and analytical methods reported for felbamate; these include high-performance liquid chromatography (HPLC) [10–14], hyphenated technique (LC–MS) [15], gas chromatography [16] and capillary electrophoresis [12]. There is a stability-indicating UHPLC method for felbamate and its related impurities [17]. This paper mainly emphasizes work related to the separation of related impurities. It lacks identification of degradation products using LC–MS/MS, deduction of degradation pathway and prediction of *in-silico* ADMET properties. In the present work, we report two new degradation products formed under alkaline-stressed conditions, their characterization, prediction of mass fragmentation pathway and their *in-silico* ADME and toxicity profile.

Methods

Chemical and reagents

Felbamate was obtained as a gift sample from Cadila healthcare limited, Ahmedabad. Acetonitrile (ACN) was purchased from Qualigens, Mumbai, Maharashtra; 15% hydrogen peroxide, concentrated hydrochloric acid and ammonium formate from Molychem, Thane, Mumbai; sodium hydroxide and HPLC grade water from Finar, Ahmedabad, Gujarat. HPLC grade water was also prepared in-house by the double distillation assembly.

Instrumentation

The HPLC method development was carried out on the Jasco LC-4000 system comprising of a model MD-4010 diode array detector (DAD); a model CO-4061 column temperature controller; a model AS-4050 autosampler; and a model PU 4180 quaternary pump system. The data acquisition and control software used was Jasco Chromenav (version, 2.01.06) installed on an ACER computer

system running Microsoft Windows 7 Professional operating system. HPLC method development trials were taken on Agilent's XDB C18 column (150 mm × 4.6 mm, 5 μm) and Phenomenex C8 column (250 mm × 4.6 mm, 5 μm). The LC–MS/MS analysis was performed on Agilent's 1200 series HPLC system and 6200 series Q-TOF detector.

The other instruments used are bath sonicator (Citizon, Vadodra, India); precision balance (Wensar digital, Chennai, India); constant temperature water bath (Cintex, 326, Mumbai, India); pH meter (Labtronics, LT-10, Panchkula, India); hot air oven (Universal, Ambala, India); photostability chamber (Newtronics Lifecare Pvt. Ltd., Mumbai, India); and double distillation apparatus (Bhanu scientific instruments, Bangalore, India).

Stress studies

The guidance provided in the ICH guideline Q1A(R2) [18] was used to probe the stability of the drug. It was stressed under hydrolytic, photolytic, dry heat and oxidative conditions. The diode array detector (DAD) was set to record absorbance at 206 nm. The structure of the degradation products (DPs) was characterized by LC–MS/MS.

Felbamate solution having concentration 1000 μg mL⁻¹ was prepared in HPLC grade acetonitrile to study the effects of the hydrolytic, oxidative and photolytic conditions. The drug was sealed in a glass ampoule before being subjected to thermal stress conditions.

The drug was stressed separately under acidic, basic and neutral conditions to assess its ability to withstand hydrolytic degradation. In a calibrated 10-mL volumetric flask, 1 mL of stock solution of felbamate was pipetted. To this, 0.1 mL of the stressor (Water, 0.1 N NaOH and 0.1 N HCl) was added. It was exposed to the stress condition for 2 days. The drug was also heated at 70°C on a constant temperature water bath with the stressor for a period of 7 h to induce degradation. Following the stress period, the stress samples were neutralized by adding 0.1 N HCl or 0.1 N NaOH, respectively. The volume was made up to mark with mobile phase and injected and run under optimized chromatographic conditions.

Oxidative degradation was carried out using 15% hydrogen peroxide as the oxidant. In a calibrated 10-mL volumetric flask, 1 mL of stock solution of felbamate was pipetted. To this, 0.1 mL of 15% hydrogen peroxide was added. It was exposed to the oxidant for a period of 2 days. To induce oxidative degradation, the drug was separately heated with 15% hydrogen peroxide for a period of 7 h. After the exposure period, the volume was made up to mark with mobile phase and injected and run under optimized chromatographic conditions.

For thermal degradation, a sealed glass ampoule containing the drug was placed inside a hot air oven at a temperature of 70 °C for a period of 7 days. After the exposure, the drug sample was injected and run under optimized chromatographic conditions.

Photolytic degradation was carried out in the solid and liquid states. The solid drug sample was dispersed as a thin layer in Petri dish, and 10 mL of standard stock solution of drug (1000 µg mL⁻¹) placed in the volumetric flask was exposed to sunlight for 7 days and ICH recommended dose of light in photostability chamber. After the exposure, appropriate dilutions were made using mobile phase to get a predetermined drug concentration of (100 µg mL⁻¹). The samples were injected in HPLC for analysis.

The stress conditions are summarized in Table 1.

Preparation of samples for HPLC analysis

Felbamate stock solution having a concentration of 1000 µg mL⁻¹ was prepared by transferring 50 mg of it in 50-mL volumetric flask. To this 10 mL of acetonitrile was added. The contents of the flask were sonicated for a period of 5 min with occasional shaking. The volume was made to mark with acetonitrile.

The mobile phase was prepared by mixing ammonium formate (10 mM, pH adjusted to 3.7 with formic acid) with HPLC grade acetonitrile in the ratio of 80:20 (v/v).

Table 1 Stress conditions for the degradation study of felbamate

Stressors	The concentration of the stressor	Exposure condition and duration
<i>Hydrolytic degradation</i>		
Hydrochloric acid	0.1 N	(1) Room temperature for 2 days (2) Heat at 70 °C for 7 h
Sodium hydroxide	0.1 N	(1) Room temperature for 2 days (2) Heat at 70 °C for 7 h
Water	-	(1) Room temperature for 2 days (2) Heat at 70 °C for 7 h
<i>Oxidative degradation</i>		
Hydrogen peroxide	15%	(1) Room temperature for 2 days (2) Heat at 70 °C for 7 h
<i>Thermal degradation</i>		
Hot air oven	70 °C	7 days
<i>Photolytic degradation</i>		
Sunlight	-	(1) 7 days (2) Fluorescence light 1.2 million lux hours and UV light 200 whm ⁻²

The stressed drug samples exposed to conditions mentioned in Table 1 were diluted with the mobile phase mentioned above to produce a concentration of 100 µg mL⁻¹ of felbamate.

HPLC method development

The pKa of felbamate was calculated to be 14.98 by Marvin Sketch (Ver 19.9.0, 2019, www.chemaxon.com). According to the literature, the most widely used column for HPLC method development of felbamate was C18. As for the mobile phase, acetonitrile and methanol were used in combination with buffers (phosphate, acetate) having pH in the range of 3.5 to 7. The preferred mode of elution was isocratic. In the present study, attempts were made to develop HPLC method using a combination of ammonium formate buffer having a concentration of 10 mM (pH adjusted to 3.7 with formic acid) and acetonitrile. Trials were taken with Agilent's XDB C 18 column (150 mm × 4.6 mm, 5 µm) and Phenomenex C 8 column (250 mm × 4.6 mm, 5 µm). The diode array detector was set to scan from 400-200 nm to determine the wavelength for maximum absorbance (λ_{max}).

HPLC method validation

The HPLC method developed was validated in accordance with the recommendation in the ICH Q2 guideline [19].

Specificity and selectivity

The specificity of the HPLC method was determined by the separation obtained between the drug peak and the DPs and in between the DPs. The separation between the peaks was expressed in terms of resolution. As for the selectivity, it was established by the peak purity value calculated through the data acquisition software.

Linearity and range

Linearity of the HPLC method in the concentration range of 20–120 µg mL⁻¹ was established. At each selected concentration level, the sample was injected in triplicate and the average peak area was calculated. The data obtained was analyzed by using regression analysis.

Precision

The precision of the HPLC method was determined in terms of intraday and inter-day precision. Precision studies were carried out at three concentration levels (40, 50 and 60 µg mL⁻¹). The precision of the HPLC method was expressed by calculating the standard deviation and the % coefficient of variation (%CV).

Accuracy

The accuracy of the HPLC method was expressed in terms of % recovery. The standard addition method was employed to calculate the recovery. A known concentration of drugs at 40, 50 and 60 $\mu\text{g mL}^{-1}$ was separately spiked with drug degradation products. At each concentration level, samples were injected in triplicate.

LC-MS/MS studies on drug and the degradation products

Electrospray ionization in positive ion mode was used to record the mass spectrum of the drug and the degradation products. Mass range in between 100 and 1000 was scanned. The optimized MS setting used for LC-MS/MS studies of the drug and the degradation products are as follows: Vcap, 3100; fragmentor, 150; skimmer, 45; Octopole RF peak, 750; drying gas temperature, 270; drying gas flow, 11(l/min); nebulizer gas, 25psi; sheath gas temp, 295; and sheath gas flow, 10(l/min). The drying gas, sheath gas and the nebulizing gas used were nitrogen.

In-Silico toxicity studies

The drug and its degradation products were tested *in-silico* for absorption, distribution, metabolism, excretion and toxicity (ADMET). pkCSM [20] web server was used to calculate ADMET. Further screening was carried out using OSIRIS property explorer [21] and Toxtree [22].

Results

Drug degradation behavior

The drug exhibited hydrolytic degradation in the presence of 0.1 N NaOH, kept at room temperature for 2 days to generate two degradation products (DPs) as represented in Fig. 2 (DP1 and DP2). To further study

the formation of the degradation products, the drug was heated with 0.1 N NaOH at 70 °C. It was observed that after 1 h, the drug degrades substantially to form higher amounts of DP2 in comparison to DP1. After 2 h, complete drug degradation is observed leading to formation of DP1.

The drug was found to be stable in other conditions mentioned in Table 1.

Development and optimization of the stability-indicating assay method

The optimized HPLC method was developed on a Phenomenex C8 column (250 mm \times 4.6 mm, 5 μm). The buffer selected was ammonium formate, the pH of which was adjusted to 3.7 with formic acid. Isocratic elution having a composition of ACN: Buffer (20:80, v/v) resulted in the best separation. The λ_{max} (maximum absorbance) for the drug was found to be at 206 nm.

Table 2 Retention time, RRT and peak purity for the drug and the degradation products

Drug/ degradation products	Retention time (RT) (min)	Relative retention time (RRT)	Peak purity (>99%)
DP-1	5.3	0.50	99.38%
DP-2	7.4	0.70	99.44%
Felbamate	10.5	1.00	99.58%

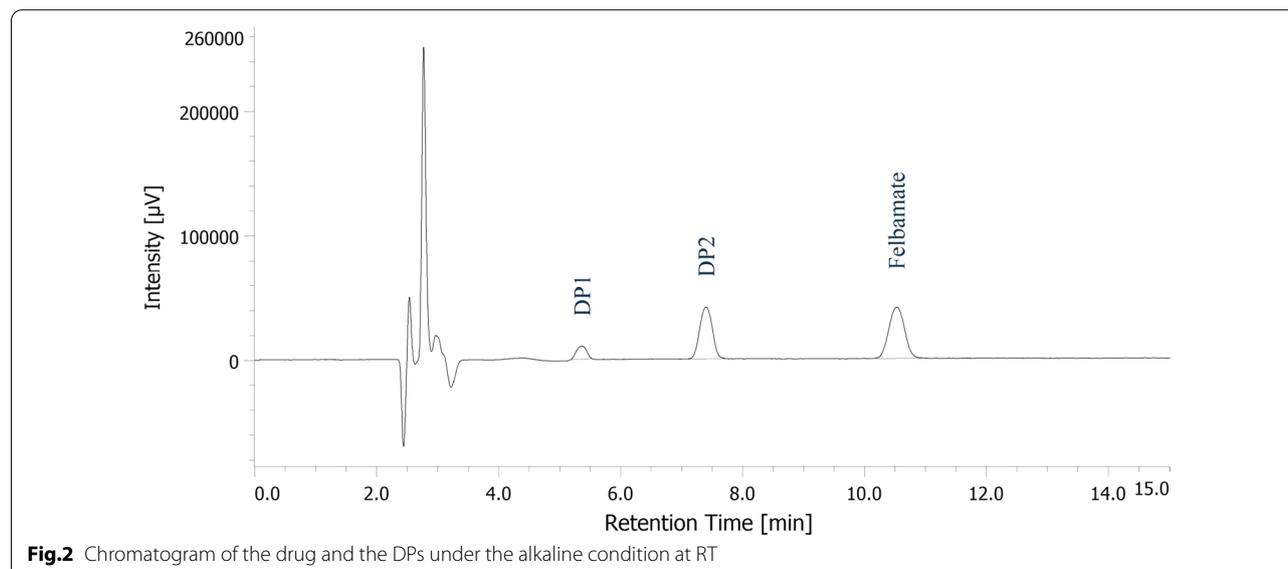


Fig. 2 Chromatogram of the drug and the DPs under the alkaline condition at RT

Table 3 Summary of HPLC method validation parameters

Validation parameters	Results
Linearity	
Equation of the regression line	* $y = 12947x - 26459$
r^2	0.9991
Range ($\mu\text{g mL}^{-1}$)	20–120
Precision	
Intraday	
$\mu\text{g mL}^{-1}$	Mean peak area \pm SD; %CV
40	489,900 \pm 1353, 0.3
50	631,412 \pm 4068, 0.4
60	750,941 \pm 10,430, 1.4
Inter-day	
40	491,919 \pm 10,430, 0.3
50	633,906 \pm 9433, 1.5
60	743,905 \pm 4482, 0.6
Accuracy	
Spiked concentration	
$\mu\text{g mL}^{-1}$	% recovery mean \pm SD, %CV
40	40.4 \pm 0.4, 1.0
50	49.4 \pm 0.6, 1.3
60	60.4 \pm 0.5, 0.9

*Where x is concentration in $\mu\text{g mL}^{-1}$ and y is the peak area

Validation of the Stability indicating assay method

Specificity and selectivity

There was no interference from the blank at the retention time of the drug or the degradation products. The blank was subjected to the same treatment as the drug before injecting it into the HPLC system. The retention time and

the peak purity (refer figure 2 of Additional file 1) are as shown in Table 2

Linearity and range

The HPLC method was determined to be linear in the concentration range of 20–120 $\mu\text{g mL}^{-1}$. The slope and the correlation coefficient (r^2) value were found to be 12,947 and 0.9991, respectively (refer figure 1 of Additional file 1).

Precision

The HPLC method precision studies (intraday and inter-day) were performed at 40, 50 and 60 $\mu\text{g mL}^{-1}$. The % coefficient of variation was found to be less than 2. This indicated that the HPLC method had sufficient reproducibility.

Recovery/accuracy

The recovery studies were carried out at 40, 50 and 60 $\mu\text{g mL}^{-1}$. The average recovery at each concentration level was found to be in-between 98 and 101%. The mean recovery was 100.2%. The chromatogram of the recovery study carried out at 60 $\mu\text{g mL}^{-1}$ is shown in Fig. 3.

The results of all the validation parameters are summarized in Table 3.

Degradation products characterization

The drugs and the DPs were studied by LC–MS/MS analysis. The data obtained were used to construct fragmentation pathways. The LC–MS and LC–MS/

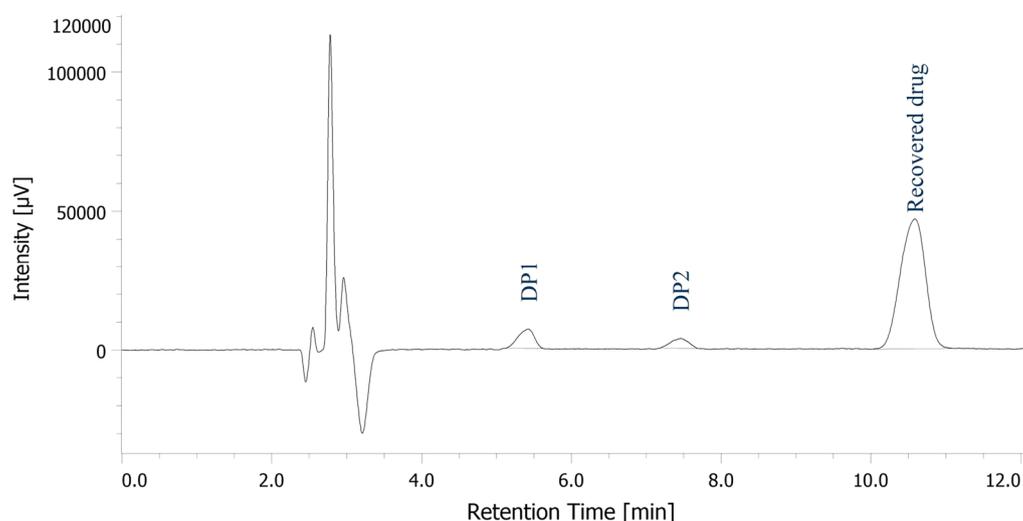
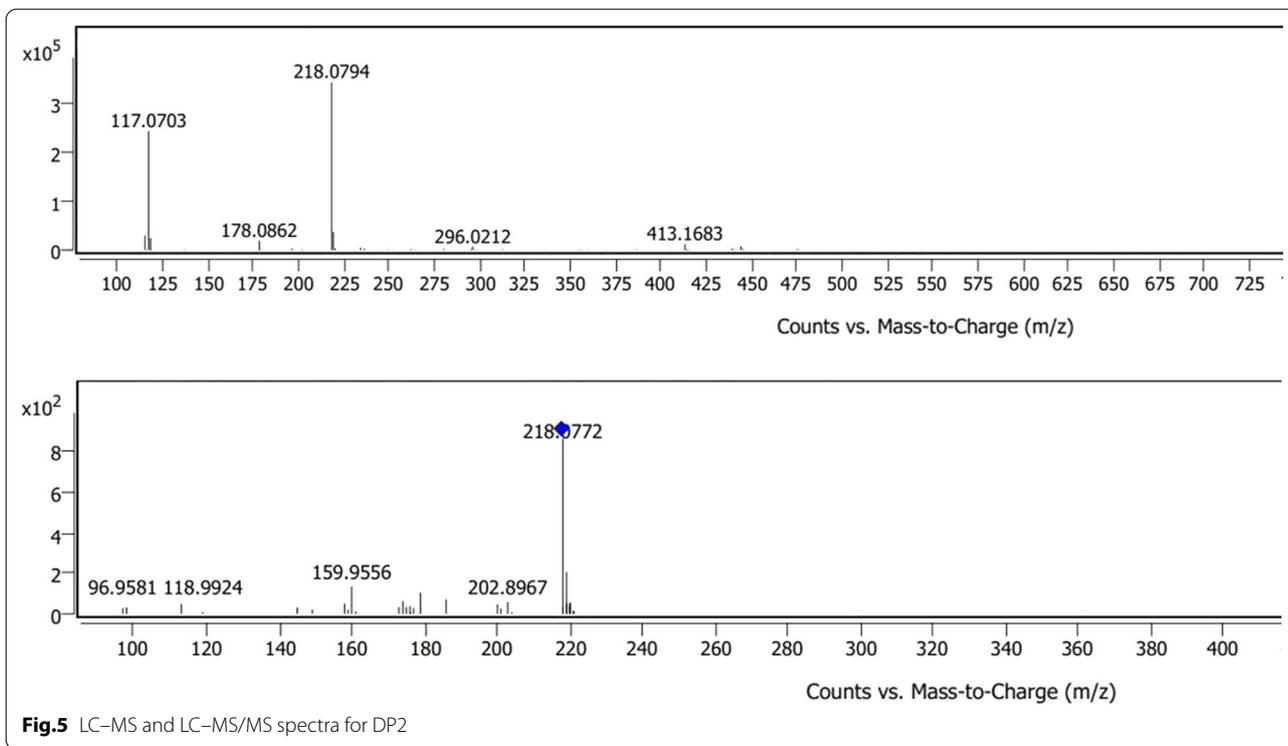
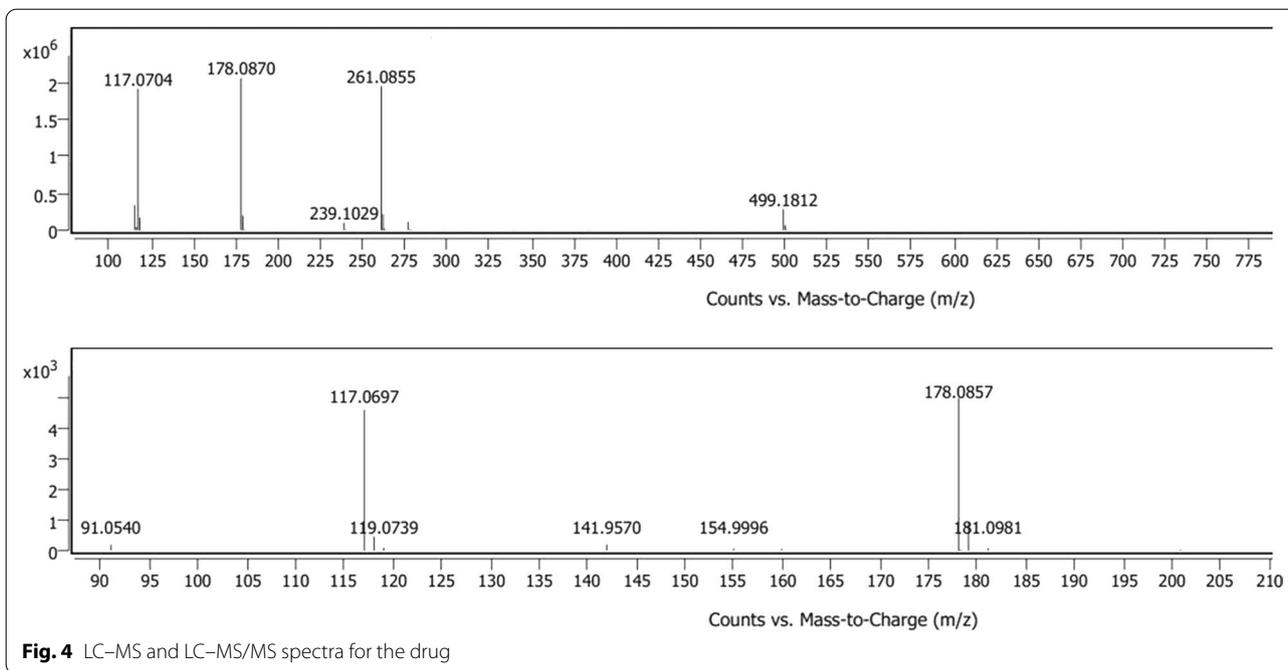


Fig. 3 Chromatogram of the recovery study carried out at a spiked concentration of 120 $\mu\text{g mL}^{-1}$

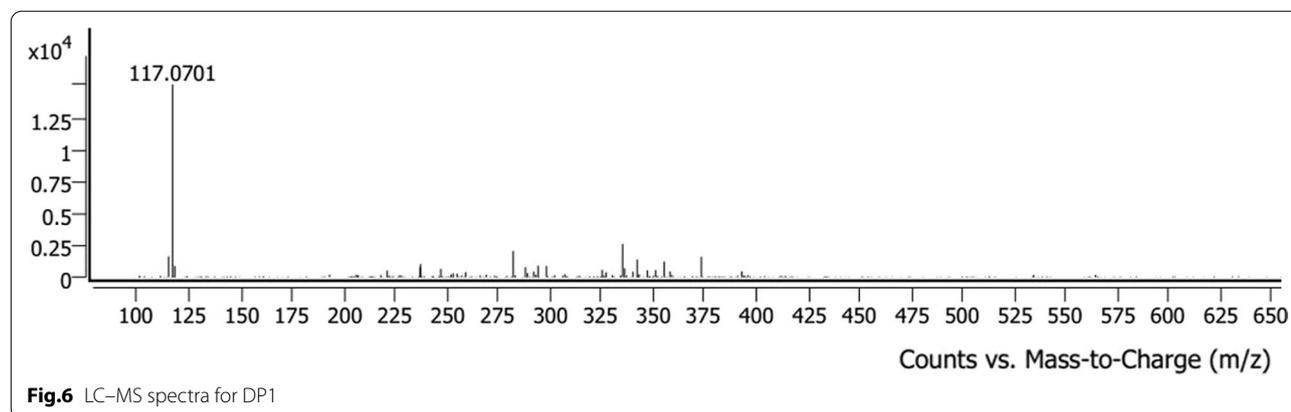


MS spectra for the drug and DP2 are as shown in Fig. 4 and Fig. 5. The LC-MS of DP1 is as depicted in Fig. 6.

The LC-MS/MS data of the drug and the DP's is represented in Table 4.

In-silico ADMET studies

The ADMET properties of the Drug and the DPs were calculated by using the pkCSM webserver. To ratify the potential mutagenic alarms, they were further screened by using Toxtree and OSIRIS property explorer. The prediction for ADMET properties by pkCSM is reported in Table 5

**Table 4** LC-MS/MS data for the drug and the DPs

Compound	Experimental mass	Most likely molecular formulae	Theoretical mass	RDB	Major fragments		Most likely molecular formulae for fragments
					Experimental mass	Theoretical mass	
Felbamate	239.1029	$C_{11}H_{15}N_2O_4^+$	239.1026	5.5	178.0857	178.0863	$C_{10}H_{12}NO_2^+$
					117.0697	117.0699	$C_9H_9^+$
DP-2	218.0794	$C_{11}H_8NO_4^+$	218.0448	8.5	202.8967	202.0499	$C_{11}H_8NO_3^+$
					159.9556	160.0757	$C_{10}H_{10}NO^+$
					118.9924	117.0699	$C_9H_9^+$
					96.9581	97.1012	$C_7H_{13}^+$
DP-1	117.0701	$C_9H_9^+$	117.0699	5.5	-	-	-

Table 5 ADMET predictions by pkCSM

Compound	Intestinal absorption (%absorbed)	PgP Substrate	BBB Permeability (log BB)	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 Inhibitor	CYP2D6 Inhibitor	CYP3A4 Inhibitor	AMES toxicity	Hepatotoxicity
Felbamate	67.85	No	- 0.206	No	No	No	No	No	No	Yes
DP1	96.10	No	0.516	Yes	No	No	No	No	No	No
DP2	95.16	No	0.125	Yes	No	No	No	Yes	Yes	No

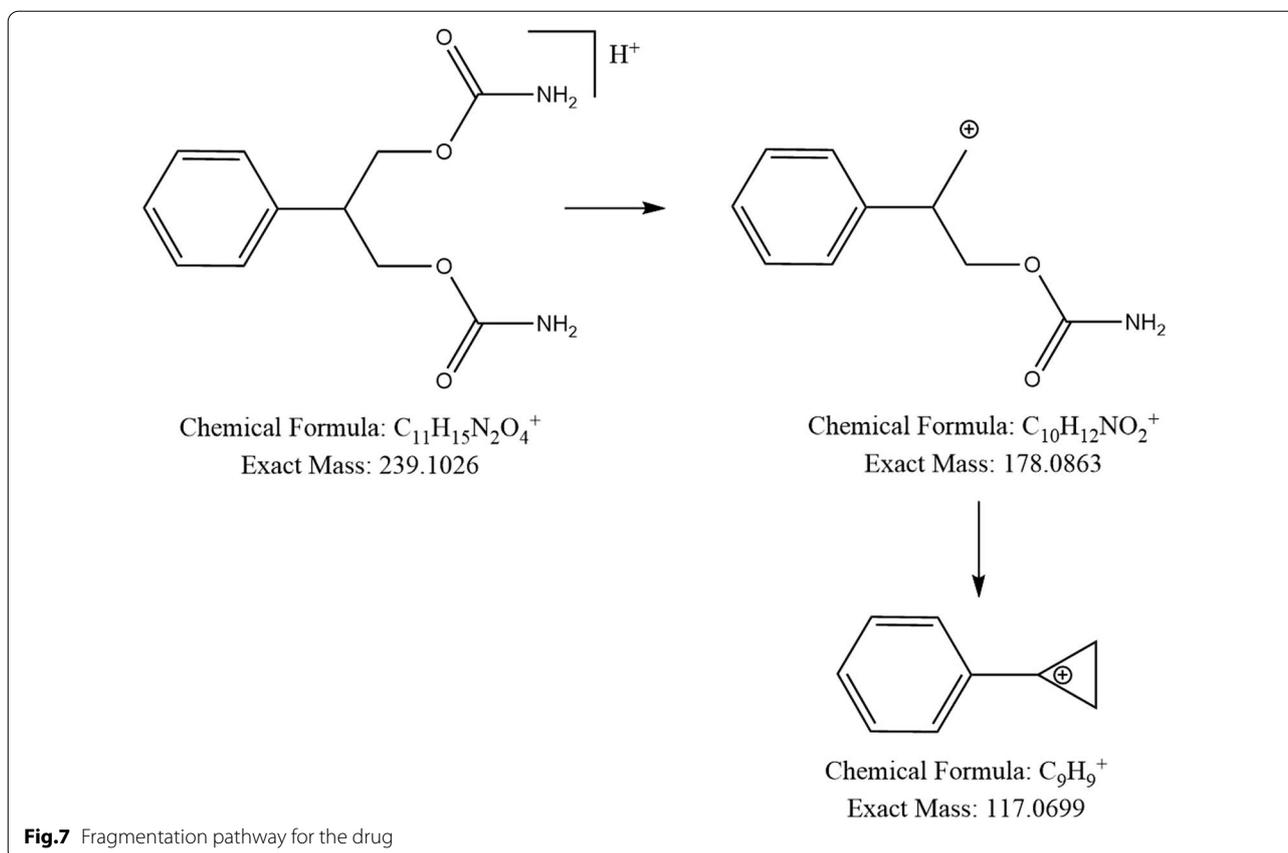
Discussion

Characterization of degradation products

Felbamate has an exact mass of 238.0953. In the LC-MS spectra recorded in positive mode of electrospray ionization (+ESI) showed a molecular ion peak $(M+H)^+$ at $m/z=239.1026$. The presence of the molecular peak ion was also confirmed by the presence of an additional peak at $m/z=261.0845$ formed due to sodium adduct $(M+Na)^+$. The drug forms two fragments having m/z value of 178.0868 and 117.0702. Fragment having m/z of 178.0868 was formed due to loss of carbamic acid fragment (exact mass=61.0163). This fragment further loses another carbamic acid moiety to generate a secondary fragment having $m/z=117.07$. The fragmentation pattern for the drug is as per Fig. 7.

Degradation product 2

The molecular ion peak for DP2 was at m/z 218.04. The formation of DP2 might be due to partial hydrolysis of the carbamate moieties followed by the subsequent cyclization. The proposed structure for DP2 and its fragmentation pathway is as shown in Fig. 8. DP2 produces four fragments having m/z value of 202.8967, 159.9556, 118.9924 and 96.9581, respectively. DP-2 loses oxygen to yield a fragment having $m/z=202.04$. This fragment in the subsequent step loses CO_2 to form a secondary fragment having m/z value of 159.9556. The fragment with m/z value of 118.9924 is formed due to loss of 1,2-oxazirine ring from the fragment having m/z value of 159.9556. The last fragment having m/z value of 96.9581 was formed



from the fragment of $m/z = 118.9924$ due to conversion of phenylcyclopropane ring to cycloheptane ring.

Degradation product 1

The molecular ion peak for DP1 was seen at m/z value of 117.07. This value matched with the fragment formed from the drug. Hence, it was proposed that the structure of DP2 was similar to the fragment at $m/z = 117.07$. DP1 is formed most likely due to the complete hydrolysis of the carbamate moieties followed by cyclization.

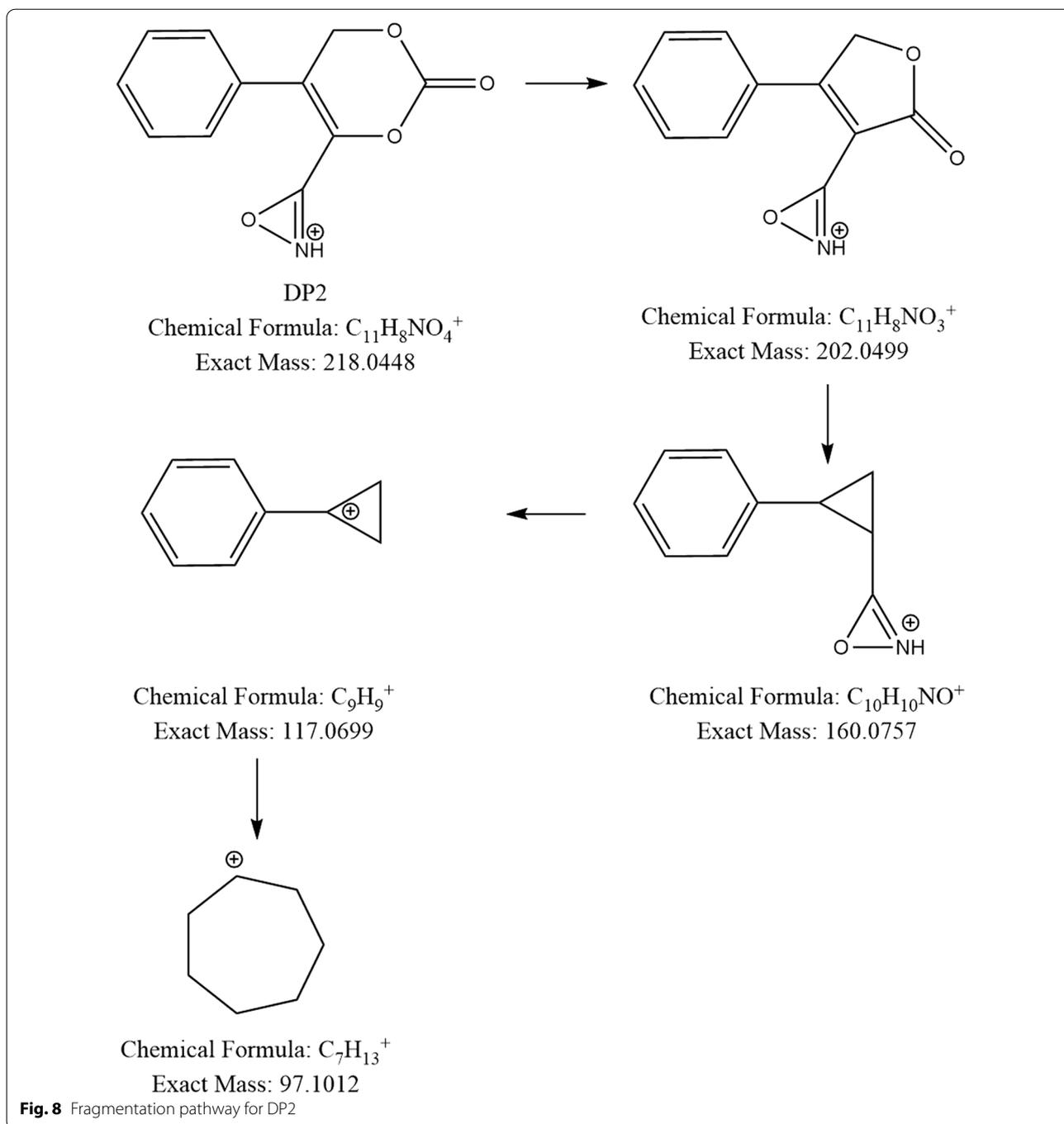
In-Silico toxicity studies

The pkCSM web server predicts the ADMET parameters based on the compound's general properties, viz., molecular properties, toxophores and pharmacophores. It also uses a distance-based graph signature to arrive at a prediction. Felbamate showed intestinal absorption of

67.85%, while both degradation products had absorption greater than 90%. Felbamate was predicted to cause hepatotoxicity. DP-1 was predicted to inhibit CYP1A2, while DP-2 as an inhibitor of both CYP1A2 and CYP3A4. Further, DP2 was flagged as a mutagenic.

To further confirm the mutagenic potential, the drug and the DPs were screened through Toxtree. Toxtree uses the decision tree approach to predict genotoxic potential. It cleared DP-1 and DP-2 for mutagenicity. However, it raised an alarm (mutagenic) for felbamate. This was attributed to the presence of the alkyl carbamate in the structure.

Due to the slight contradiction in predictions for mutagenicity, the drug and DPs were screened through OSIRIS property explorer. OSIRIS acquitted the drug and the DPs for mutagenicity. Further, it indicated that they do not possess any tumorigenic, irritant and reproductive risks (refer the Additional file 2).



Conclusions

The drug was found to be susceptible to hydrolysis in the presence of alkali. The drug exhibited resilience to degradation under other stress conditions like acidic, neutral, thermal, photolytic and oxidative. There was the formation of two DPs under alkaline stress conditions. The DPs were well resolved in the developed HPLC method. The

structural characterization of the impurities was performed by LC-MS/MS studies. The present work has revealed some novel information pertaining to: (a) the degradation profile of felbamate under stress conditions, (b) fragmentation pathways for the DPs and (c) ADMET properties of the drug and the DPs.

Abbreviations

HPLC: High-performance liquid chromatography; DPs: Degradation products; LC–MS/MS: Liquid chromatography-mass spectrometry; RDB: Ring double bond equivalent; RRT: Relative retention time; RT: Retention time; ADMET: Absorption, distribution, metabolism, excretion and toxicity; CYP1A2: Cytochrome P450 family 1 subfamily A member 2; CYP3A4: Cytochrome P450 family 1 subfamily A member 4.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s43094-021-00347-8>.

Additional file 1. Supplementary information about the linearity study and the peak purity data.

Additional file 2. Supplementary information for toxicity evaluation of felbamate and its degradation products (DPs).

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Authors' contributions

RSC and AAM contributed to conceptualization, plan of work, LC–MS study, toxicological study and writing and review of original draft; RSC contributed to analysis and investigation; AAM contributed to supervision. All authors have read and approved the final manuscript.

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Availability of data and materials

Data and material will be available on request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they do not have competing interests.

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