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Degradation studies of pentoxifylline: Isolation and characterization of a novel *gem*-dihydroperoxide derivative as major oxidative degradation product

Mahesh Kumar Mone[a,b,*], K.B. Chandrasekhar[b]

[a] Drug Discovery Facility, Advinus Therapeutics, Quantum Towers, Plot-9, Rajiv Gandhi Infotech Park, Phase-I, Hinjewadi, Pune 411057, India
[b] Department of Chemistry, Jawaharlal Nehru Technological University, College of Engineering, Anantapur 515002, India

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ABSTRACT

Pentoxifylline was subjected to various stress conditions and degradation profile was studied with conventional LCMS. Interestingly, under oxidative stress conditions the drug substance underwent distinct transformation to give rise to a single major degradation product. The structure of this product was elucidated using 1D, 2D NMR spectroscopy, high resolution mass spectrometry (Q-TOF LC/MS) and found to be a novel *gem*-dihydroperoxide, namely 1-(5,5-Bis-hydroperoxy-hexyl)-3,7-dimethyl-3,7-dihydro-purine-2,6-dione. An efficient stability indicating liquid chromatographic separation method was developed for pentoxifylline and its three degradation products (including two from base hydrolysis) using 1.8 μm, C18 reverse phase column and UHPLC. Baseline separation was achieved with a run time of 4 min. The analytical assay method was validated with respect to system suitability, specificity, linearity, range, precision, accuracy and robustness.

1. Introduction

Pentoxifylline (3,7-dimethyl-1-(5-oxohexyl)-3,7-dihydro-1*H*-purine-2,6-dione) is a synthetic dimethyl xanthine derivative structurally related to theophylline and caffeine (Fig. 1). It is a non-selective phosphodiesterase inhibitor which increases erythrocyte cAMP activity and allows the erythrocyte membrane to maintain its integrity as well as resistance to deformity. It is used for the treatment of peripheral vascular diseases, management of cerebrovascular insufficiency, sickle cell disease, and diabetic neuropathy [1–3]. Subsequent investigations have also revealed that pentoxifylline improves sperm motility both *in vitro* and *in vivo* [4].

Numerous analytical methods were reported for the analysis and estimation of pentoxifylline based on diverse analytical instrumentation and methodologies. These include SFC method [5], HPTLC method [6], HPLC reverse phase methods using various particle size columns and buffers [7–10]. The drug was also studied extensively for pharmacokinetics and metabolism in animals and human subjects leading to development of a number of HPLC methods using UV and mass detection [11–13]. There is a recent report on thermal and chemical stability of the formulation aimed to study drug release and stability [14]. The solution stability under alkaline conditions and thermal stability in solid state along with stability under day light were previously reported [15]. Wherein the degradation was monitored by TLC and the two base hydrolysis products were identified. These two alkaline hydrolysis products were later synthesized and characterized [16]. However, to the best of our knowledge, no report has been published on pentoxifylline and its degradation under oxidative stress condition. Hence, we decided to study the degradation pattern of pentoxifylline under oxidative stress conditions as it has an interesting chemical structure with xanthine scaffold and a side chain with a keto functionality that has extended from N1 of xanthine. We have also conducted degradation studies under alkaline conditions to isolate and characterize the already reported degradation products in order to develop a stability indicating global ultra high performance liquid chromatographic (UHPLC) method that would include all major degradation products of pentoxifylline. Herein, we report (i) degradation behavior of the drug under oxidative stress and various other conditions monitored by LCMS, (ii) isolation and purification of major degradation products from stress samples including two reported base hydrolysis products (degradation product 1 and 2), (iii) characterization of all major degradation products using NMR and Q-TOF LC/MS and (iv) development and validation of a stability indicating faster UHPLC assay method suitable to be used with LCMS systems.

* Corresponding author at: Drug Discovery Facility, Advinus Therapeutics, Quantum Towers, Plot-9, Rajiv Gandhi Infotech Park, Phase-I, Hinjewadi, Pune 411 057, India. Tel.: +91 20 66539629; fax: +91 20 66539620.
E-mail addresses: mahesh.mone@advinus.com, maheshmone@yahoo.com (M.K. Mone).

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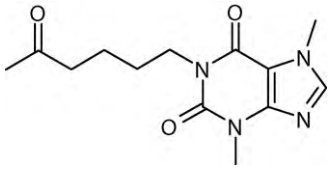

Fig. 1. Chemical structure of pentoxifylline.

2. Experimental

2.1. Drug substance and reagents

Highly pure sample of pentoxifylline (total impurities not more than 0.03%) was received as a gift sample from Hikal Ltd., Bangalore, India. Ultra high pure Water was obtained from in-house Millipore Milli Q water system and was used for the preparation of solutions and their dilutions. Acetonitrile was purchased from JT Baker, USA. Sodium hydroxide, hydrogen peroxide, hydrochloric acid, methanol were procured from Merck. Formic acid was procured from Fluka.

2.2. Instrumentation and equipment

Initial HPLC separations were carried out using Agilent 1200 Series HPLC system equipped with diode array detector (G1315B), quaternary pump (G1311A), an on-line degasser (G1322A), an auto-sampler (G1367B) with thermostat (G1330B) and a column thermostat (G1316A). UHPLC–MS method development was done on Agilent 1200 Series rapid resolution liquid chromatograph (RRLC) with 80 Hz diode array detector (G1315C), a binary pump (G1312B) with degasser (G1322A), an auto-sampler (G1329B) with thermostat (G1330B), a column compartment (G1316B) and a single quadrupole mass spectrometer (G6110A) with electro spray ionization source. Purification of the degradation products was carried out using Agilent 1200 Series preparative HPLC system consisting of two pumps (G1361A) capable of running in gradient mode, a multiple wavelength detector (G1365B), an auto-fraction collector (G1364C) and an auto-sampler (G2260A).

The NMR spectral data was generated using Varian NMR 400 MHz (^{1}H, 400 MHz; ^{13}C, 100 MHz) instrument with a dual broad band probe and z-axis gradients. High resolution mass spectrometric analysis was carried out on Agilent 6520 Accurate-Mass Q-TOF LC/MS. The infrared spectroscopy data were generated on Perkin Elmer FT-IR spectrum 100.

The stress studies were conducted in thermal stability chambers from Binder (KBF 720). The photostability chamber is also from Binder (KBF 720-ICH-LIGHT). Purified samples were dried in freeze dryer model ModulyoD (Thermo Electron Corporation).

2.3. Forced degradation studies

Forced degradation studies were carried out under different stress conditions following procedures reported in literature [17–19].

Exposure to light: Solid drug substance as a thin layer (~1 mm) was exposed to ultra violet light of 254 nm at 7614 lx h for 10 days equivalent to 1.8 million lx h unlike procedure reported earlier [15].

Oxidative stress condition: Oxidative stress study was carried out by dissolving 2 and 20 mg ml^{-1} concentrations of pentoxifylline in 3% and 30% hydrogen peroxide solutions respectively at different temperatures (room temperature (~25 °C), 40 and 80 °C) with varied exposure time.

Acidic and alkaline stress condition: Acidic and alkaline stress studies were carried out by dissolving pentoxifylline at 1 and 2 mg ml^{-1} concentrations in 0.1N HCl and 0.1N NaOH respectively, at room temperature (25 °C), 40 and 80 °C with varied exposure time.

2.4. Liquid chromatographic separation studies

2.4.1. Development of stability indicating HPLC, UHPLC methods for degradation products profiling

Initial analysis of the degradation samples for profiling was performed with conventional HPLC system with Zorbax XDB C18 column (250 mm × 4.6 mm 5 μm) kept at 40 °C, using a gradient program comprising 0.05% formic acid in water (A) and acetonitrile (B). Gradient elution was carried out at constant flow of 1 ml min^{-1}, from 95%A to 0%A (corresponding to 5%B to 100%B) for 10 min, followed by an isocratic elution at 100%B for 3 min. The gradient was brought back to initial conditions of 95%A and 5%B in a minute and the system was stabilized for 3 min. In all the cases, a photodiode array detector was used along with mass detector to ensure complete separation. Chromatograms were acquired at 274 nm.

Further, in an effort to reduce run time, UHPLC method was also developed using Zorbax column (Eclipse XDB-C18 Rapid Resolution HT 4.6 mm × 50 mm 1.8 μm, maximum pressure tolerance: 600 bar) on an Agilent RRLC system equipped with a PDA detector and MS detector.

2.4.2. Validation of UHPLC assay method

UHPLC–MS method was validated with respect to system suitability, specificity, linearity, range, precision, accuracy and robustness. Linearity, LOD and LOQ were determined for degradation product 3. All the solutions and dilutions were prepared in water except for degradation products-3 where in the initial stock solution was prepared in methanol, and subsequent dilutions were prepared with water.

System suitability: The system suitability was checked by making five replicate injections of a mixture of 1 mg ml^{-1} degradation solution from oxidative stress and alkaline stress hydrolysis samples of pentoxifylline in equal volumes. The resolution between peaks is measured by chemstation software.

Specificity: The specificity of the UHPLC method for pentoxifylline was determined in presence of its closely eluting degradation product. For this purpose degradation product 3 and pentoxifylline were mixed at 1 mg ml^{-1} concentration and chromatographed. Peak purity of the drug was checked by diode array detector to rule out overlap of peaks.

Linearity and range: Linearity test solutions were prepared from drug stock solution of 1 mg ml^{-1} in water at six concentrations, viz., 50, 100, 200, 300, 400, and 500 μg ml^{-1} in triplicate. Each solution was analyzed injecting 5 μl. The experiments were repeated on two consecutive days. The measured areas were used to plot linearity curve and to calculate RSD. Further the linearity has been evaluated by the residuals of the regression line. Similar procedure was applied to degradation product 3 to establish linearity in the same concentration range.

Precision: Precision test solutions were prepared from drug stock solution of 1 mg ml^{-1} in water at three concentrations, viz., 100, 300, 500 μg ml^{-1} analyzed six times on the same day and three consecutive days. The experiment was repeated by a different analyst on day four.

Accuracy: To establish accuracy, recovery studies were carried out by spiking known standard samples with stress sample. Three standard solutions were prepared in duplicate at 100, 200 and 300 μg ml^{-1} spiked with fixed volumes of stress sample. After injecting standard linearity solutions, stress sample and the spiked standards were analyzed. The percentage of recoveries was calculated from the slope and *y*-intercept of the calibration curve.

In addition, accuracy of the assay method was evaluated by estimating the pentoxifylline content in standard formulation. Trental® 400 (400 mg per tablet) sustained release tablet one each in duplicate was crushed to fine powder and transferred quantitatively to a beaker with water and ultrasonicated for 3 h, cooled and

transferred to 200 ml volumetric flask.100 ml of this solution is further diluted 200 ml. 50 ml of each sample solution is further diluted to 200 ml with water. This solution is filtered through 0.45 μ membrane filters before injection. After linearity check and standard solution injections, sample solution was injected and percentage assay was calculated.

Limit of detection (LOD) *and limit of quantification* (LOQ): The LOD and LOQ for degradation product 3 were determined at a signal-to-noise ratio of 3:1 and 10:1 respectively, by injecting a series of solutions with known concentrations. Precision at LOQ level was determined for multiple injections ($n = 6$) by calculating %RSD of the area.

Robustness: Robustness is the capacity of an analytical method to remain unaffected by small, deliberate variations in method parameters. The performance parameter monitored was resolution between the drug and the closely eluting degradation product. To study the effect of variation in flow rate, flow was purposely varied by 10% on lower side and higher side throughout the gradient and the resolution was measured. The effect of column temperature variation on resolution was measured at 35 and 25 °C. The variation in acid concentration in mobile phase is studied at 0.03% and 0.08% formic acid in water. The mobile phase proportions i.e. 0.05% formic acid and acetonitrile were varied throughout the gradient by 10% in each case towards lower and higher side and the resolution was recorded keeping other parameters constant.

2.5. Preparation and purification of the degradation products

2.5.1. Preparation and isolation of alkali-induced degradation products

Degradation product 1 and degradation product 2 from alkali-induced degradation solution were isolated and purified using HPLC method with purity 99.6% and 97.3% respectively.

2.5.2. Preparation and isolation of the oxidative degradation product

Accurately weighed 300 mg of pentoxifylline was dissolved in 15 ml of 30% Hydrogen peroxide. The solution was stored at room temperature for 6 days.

This solution is freeze-dried to dryness. The dry mass was dissolved in water and was purified on C18 semi-preparative HPLC column (Zorbax XDB, USA) 150 mm × 21.2 mm (i.d.), 5 μm using water (A): acetonitrile (B) as eluents and with UV detection at 274 nm. Gradient elution was carried out at constant flow of 15 ml min^{-1}, from 95%A to 70%A (corresponding to 5%B to 30%B) for 20 min, followed by an isocratic elution at 5%B for 6 min (95:5, v/v). The fraction was initially concentrated with a rotavapor at room temperature to remove acetonitrile and finally water was removed by lyophilization. The purity of degradation product 3 was 99.4% by HPLC.

2.6. MS and Q-TOF LC/MS studies

Degradation products were analyzed using Agilent single quadrupole mass spectrometer with parameters mentioned in parentheses: ionization source (+ve ESI); nebulizer pressure (nitrogen, 30 psi); gas temperature (320 °C); drying gas flow (nitrogen, 7 l min^{-1}); capillary voltage (3000 V); fragmentor voltage (70–100 V). Similarly, the parameters for Agilent Accurate-Mass Q-TOF were ionization source (+ve ESI); capillary voltage (3500 V); drying gas flow (8 l min^{-1}); drying gas temperature (320 °C); nebulizer pressure (45 psi); fragmentor voltage (150–250 V). Precursor ion scans were done on API-4000 LC/MS triple quadrupole system in positive ESI. All the values indicated as m/z correspond to $(M+H)^+$ ions.

2.7. NMR studies

For the purpose of having better correlation of the data with standard drug substance, all the sample solutions were prepared in deuterated dimethyl sulfoxide as solvent and TMS as an internal standard.

2.8. IR spectra

Infrared absorption spectrum was recorded in the form of pellets dispersed in dried KBr and was scanned in the range 4000–450 cm^{-1} with 2 cm^{-1} resolution.

2.9. Sample preparation

All sample preparations, including preparative and analytical, were done in water as all the degradation products were found to be unstable under acidic conditions.

3. Results and discussion

3.1. Study of degradation behavior

Conventional HPLC analysis method (Section 2.4) with PDA and MS detector has been used to study the degradation profile of the stress samples at regular intervals. Pentoxifylline eluted at 7.5 min well separated from the degradation products (Fig. 2).

Degradation under oxidative stress: Pentoxifylline was highly susceptible to hydrogen peroxide oxidation and was degraded completely into highly polar products within 19 h at 80 °C. However, at room temperature, the degradation was more controlled giving rise to a single product (degradation product 3), which was chosen for further isolation and characterization studies.

In all we have identified and isolated three major degradation products of pentoxifylline, two from the basic stress condition namely degradation product 1 and degradation product 2, one from oxidative stress condition namely degradation product 3. The impurities were named in the order of their elution in the reverse phase HPLC chromatography. Typical chromatograms of the profiling are as shown in Fig. 2. The degradation profile summary is as detailed in Table 1.

3.2. Structure elucidation of isolated pure degradation products

A straight forward means of achieving this was by acquiring basic spectral data by NMR, and mass spectrometry for the drug and the degradation products. We initially analyzed standard drug and recorded proton, ^{13}Carbon, gCOSY, and DEPT spectrums on NMR. Further similar set of experiments were carried out for individual purified degradation products under similar experimental parameters. High resolution mass spectrometric fragmentation study of the degradation products and the drug, were carried out, under conditions suitable enough to cause sufficient fragmentation with ESI and upfront fragmentation. Additional experiments like deuterium exchange, gHMBC and NOE experiments were performed when required. Structures of degradation product 1 and degradation product 2 were confirmed to as reported in earlier reports [15,16].

3.2.1. Structure elucidation of degradation product 3

The molecular weight of degradation product 3, based on MS analysis, was found to be 328 amu, 50 amu higher than pentoxifylline. The HRMS data confirmed the elemental composition of this product to be $C_{13}H_{20}N_4O_6$ (Table 2) indicating that 50 amu increase could account for additional three oxygen and two hydrogen atoms. ^{13}C NMR spectrum indicates no change in the number of

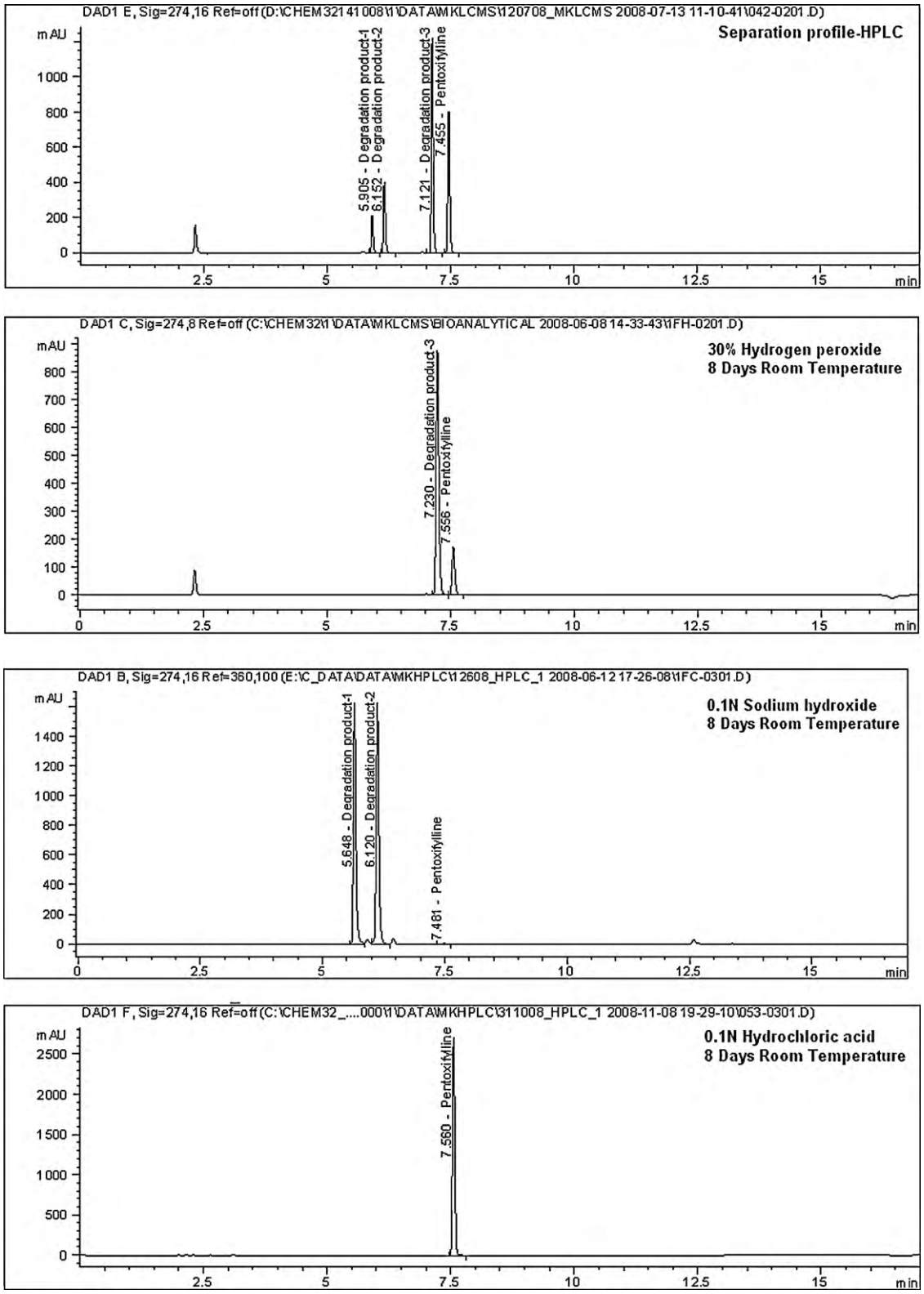

Fig. 2. HPLC chromatograms of typical degradation profiles of pentoxifylline.

carbon atoms when compared to standard pentoxifylline. The proton NMR spectrum highlights appearance of two additional protons and also considerable shift in protons attached to carbons on the either side of the carbonyl functionality (shift in methylene protons C3,H2 from 2.4 to 1.5 ppm and methyl protons C1,H3 from 2.05 to 1.2) (Table 3). In ^{13}C NMR, carbonyl carbon atom (C2) signal also shifted from 208.4 to 109.8 ppm. These shifts together confirm that there is a change in carbonyl functionality (C2).

A two proton singlet observed at 10.92 ppm that did not show any correlation in gCOSY NMR spectrum. However, these highly de-shielded protons showed correlation with terminal methyl protons (C1,H3) and also methylene (C3,H2) in 1D as well as 2D NOESY

Table 1
Summary of forced degradation profile studies.

Type of stress and duration of exposure	Area percentage observed by HPLC, confirmed by MS			
	Pentoxifylline	Degradation product 1	Degradation product 2	Degradation product 3
Light UV-254 nm/10 days	No change	ND	ND	ND
30% H_2O_2/~25 °C/20 mg ml^{-1}/8 days	14% remains	ND	ND	78%
3% H_2O_2/~25 °C/2 mg ml^{-1}/8 days	12% remains	ND	ND	75%
0.1N HCl/80 °C/2 mg ml^{-1}/4 days	No change	ND	ND	ND
0.1N HCl/~25 °C/1 mg ml^{-1}/8 days	No change	ND	ND	ND
0.1N NaOH/80 °C/2 mg ml^{-1}/2 days	6% remains	16%	74%	ND
0.1N NaOH/80 °C/2 mg ml^{-1}/4 days	ND	25%	73%	ND
0.1N NaOH/~25 °C/1 mg ml^{-1}/8 days	ND	48%	51%	ND

ND: not detected.

Table 2
Molecular formula, calculated mass for $(M+H)^+$ ions, measured accurate mass (by LC/Q-TOF), and error in ppm of pentoxifylline and degradation product 3.

Property	Products	
	Pentoxifylline standard	Degradation product 3
Molecular formula	$C_{13}H_{18}N_4O_3$	$C_{13}H_{20}N_4O_6$
Calculated mass for $(M+H)^+$	279.14587	329.14623
Measured mass m/z $(M+H)^+$	279.1455	329.1462
Error in ppm	−1.33	−0.09

studies (Fig. 3), indicating close proximity to terminal methyl (C1) and methylene (C3) groups. Deuterium exchange experiment confirmed that these two protons are readily exchangeable. gCOSY NMR spectrum shows unaltered correlations with respect to other methylene and ring protons.

The HRMS fragmentation profile is distinct from other degradation products, but shows three fragments corresponding to pentoxifylline (m/z 279) (Fig. 4a and b) and its fragments (m/z 181 and m/z 138), indicating possible reformation of pentoxifylline which in turn forms its fragments (Fig. 5 and 6a). Additional observation is that degradation product 3 in acidic solution decomposes to form pentoxifylline. A unique fragment of m/z 235 is formed by a possible loss of peroxide group as peracetic acid further confirms the proposed structure for degradation product 3 (Fig. 6b). However, a small band observed at 1394.36 cm^{-1} in IR spectrum, arising due to O–H bending of peroxide functionality [20].

Based on above mentioned observations derived from various spectral data, degradation product 3 was assigned a *gem*-dihydroperoxide structure, chemically named as 1-(5,5-Bis-hydroperoxy-hexyl)-3,7-dimethyl-3,7-dihydro-purine-2,6-dione. Fig. 7 shows the degradation pathways of pentoxifylline in base and peroxide media illustrating the structures of degradation products 1, 2 and 3. Synthesis of *gem*-dihydroperoxides is reported in the literature and these are formed by reacting keto compounds with aqueous hydrogen peroxide [21]. Dihydroperoxides may serve as intermediates for the synthesis of acyclic peroxides with anti-malarial activity [22].

3.3. Development and optimization of UHPLC method

Our aim was to develop an efficient stability indicating UHPLC method which should be compatible with LC–MS systems, as the mass spectrometry is currently the most powerful and affordable analytical tool for the qualitative identification, characterization of drug substances, related impurities and also for the quantitative estimation of drugs and their metabolites.

Table 3
Summary of proton NMR shifts and coupling constants of pentoxifylline and degradation product 3.

Proton position[a]	Pentoxifylline			Degradation product 3		
	^1H	δ (ppm)	J (Hz)	^1H	δ (ppm)	J (Hz)
1	3	2.05	s	3	1.22	s
3	2	2.45	t (6.6)	2	1.53	t (7.34)
4	2	1.46	m	2	1.73	m
5	2	1.46	m	2	1.59	m
6	2	3.83	t (7.0)	2	3.85	t (7.34)
9	3	3.86	s	3	3.87	s
10	1	8.00	s	1	8.01	s
12	3	3.40	s	3	3.41	s
14				1	10.94	s
15				1	10.94	s

[a] Refer Fig. 7.

Table 4
Final UHPLC gradient program.

Time (min)	Acetonitrile	0.05% formic acid	Flow (ml min^{-1})
0.0	10%	90%	1.0
3.0	30%	70%	2.5
3.2	10%	90%	2.5
4.0	10%	90%	2.0

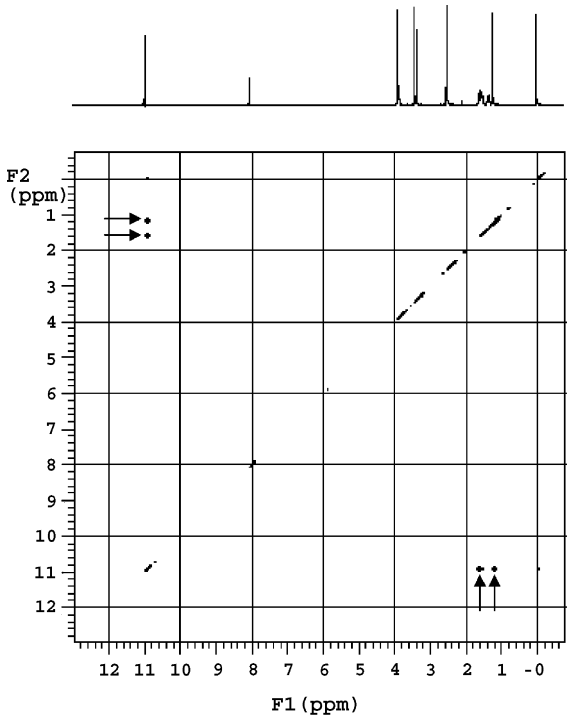

Fig. 3. 2D NOESY spectrum of degradation product 3: cross peaks identified with arrows correlating protons at 10.94 ppm (peroxide) with 1.53 ppm (methylene) and 1.22 ppm (methyl) protons.

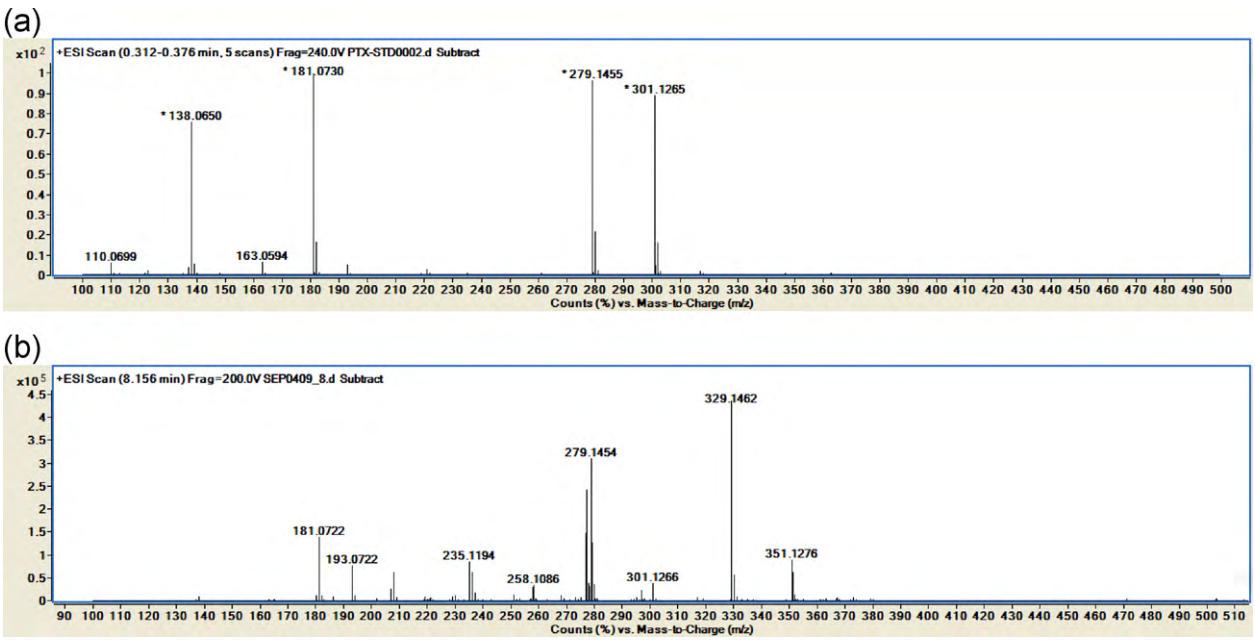

Fig. 4. LC/Q-TOF spectra of (a) pentoxifylline, (b) degradation product 3.

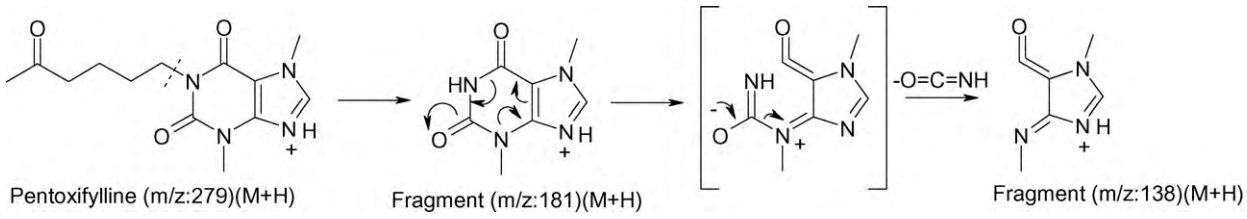

Fig. 5. Proposed mass spectrometric fragmentation pathway of pentoxifylline.

The sample used for method development was the degradation reaction mixture obtained from oxidative and alkaline stress conditions as it is likely to cover all the major degradants formed in this study. Different gradient elutions were tried with 0.05% formic acid in water and acetonitrile. Two sets of peaks were obtained in chromatograph, degradation products 1 and 2 were polar in nature when compared to product 3 and pentoxifylline. An increase in acetonitrile proportion could reduce the run time but separation between degradation product 3 and pentoxifylline had reduced considerably. Hence, the flow rate was increased linearly from 1 to 2.5 ml min^{-1}, to retain separation and decrease the run time considerably. Finally, column thermostat temperature was optimized.

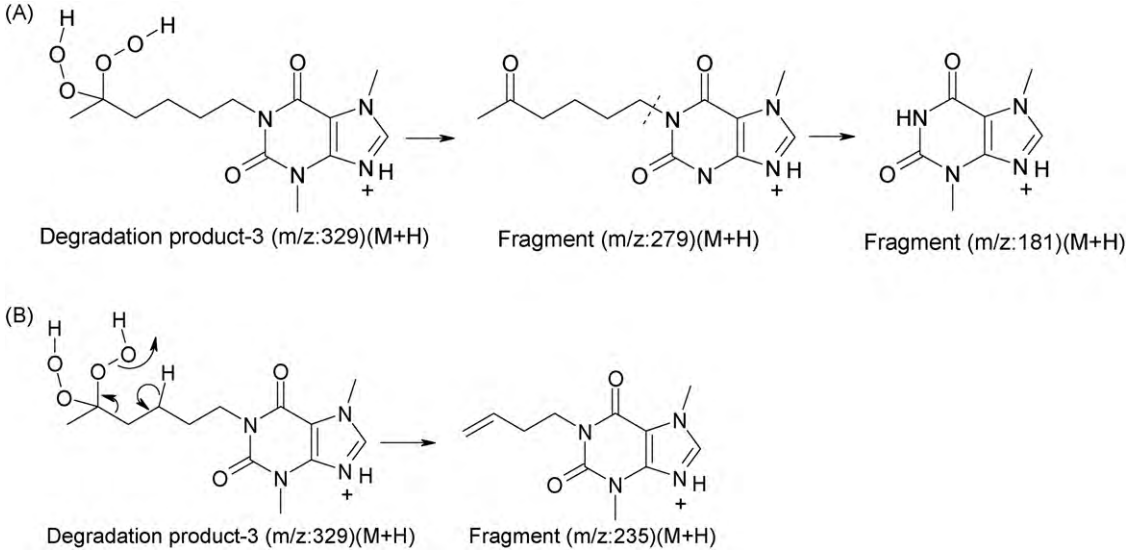

Fig. 6. Proposed mass spectrometric fragmentation pathways of degradation product 3.

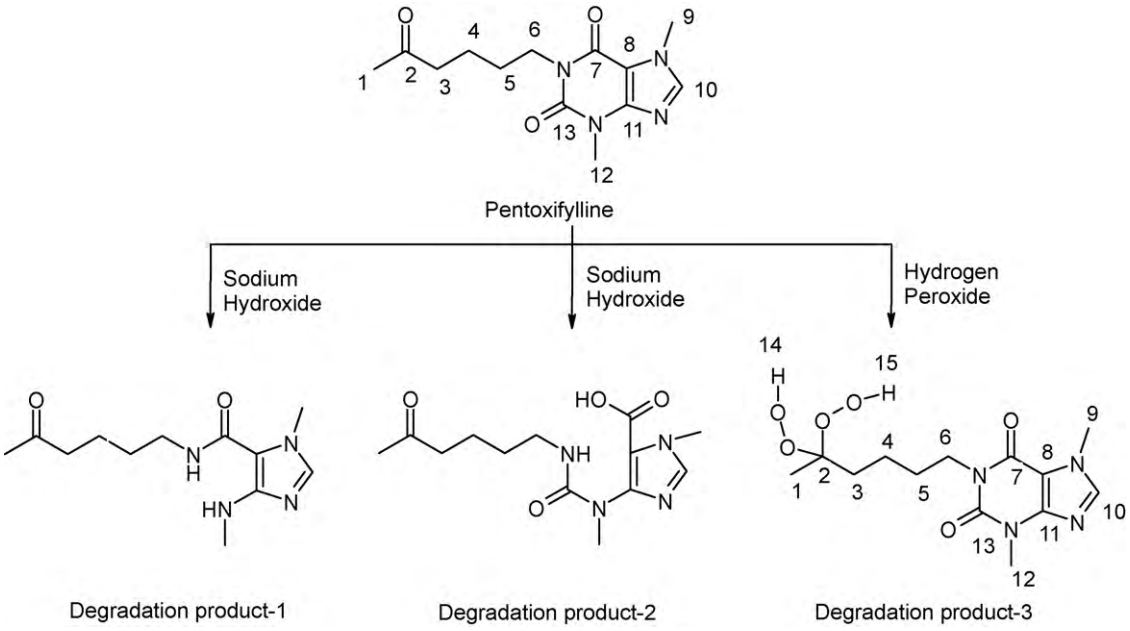

Fig. 7. Proposed degradation pathways of pentoxifylline.

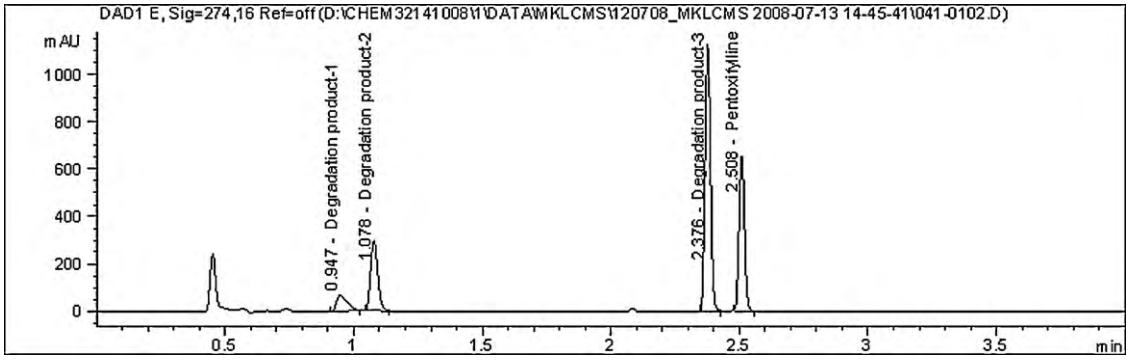

Fig. 8. UHPLC chromatogram of pentoxifylline and its degradation products.

At 30 °C, the column pressure varied from 168 bar at 1 ml min^{-1} flow rate to 415 bar at 2.5 ml min^{-1} during gradient run. The final gradient method is given in Table 4 and the separation profile is as shown in Fig. 8.

3.4. Results of UHPLC analytical method validation

The resolution factor between degradants and pentoxifylline was found to be satisfactory indicating baseline separation (Table 5, Fig. 8). The purity factor is within the threshold limit obtained in the resolution chromatogram which demonstrates the analyte peak homogeneity. The linearity of the method was established in a concentration range of 50–500 μg ml^{-1}, the response was strictly linear in the concentration range (R^2 = 0.9998) (y = 5.0857x + 5.5318). The linearity correlation coefficient for degradation product 3 was 0.9994. From the residuals, it can be concluded that most of the points are close to the linearity.

Table 5
Results of robustness study.

Method parameter	Variation	Resolution
Mobile phase flow	10% low	3.37
	10% high	3.26
Column temperature	25 °C	2.90
	35 °C	3.67
Formic acid content	0.03%	3.30
	0.08%	3.28
% Organic phase (inversely aqueous phase)	10% low	3.35
	10% high	3.22

The runs test suggests that the data does not vary significantly from linearity and hence the assumption of linear regression holds true. The RSD for precision samples (n = 6) varied from 0.03 to 0.35% for all the experiments repeated for 4 days. The percentage recovery

Table 6
Retention time variation study for 14 injections (n = 14), and resolution variation between peaks.

Product	RT variation in min	% RSD	Resolution variation	% RSD
Degradation product 1	0.945–0.952	0.22		
Degradation product 2	1.076–1.082	0.15	2.06–2.10	0.55
Degradation product 3	2.376–2.380	0.07	28.67–29.07	0.46
Pentoxifylline	2.508–2.513	0.07	3.29–3.32	0.32

of pentoxifylline from stress sample ranged from 98.2 to 98.9%. Trental® tablets formulation assay value carried out in duplicate was found to be 96.7% and 97.6%. The LOD and LOQ for degradation product 3 were found to be 0.5 and 2 $\mu g\,ml^{-1}$, respectively. The precision of degradation product 3 at the LOQ concentration is 2.6% for $n = 6$. The result of the robustness study is summarized in Table 5. Since the flow rate was varied during the gradient run we wanted to check the retention time variation and performance reproducibility for all the peaks, for multiple injections. The retention time variation was minimal and the resolution values were reproducible (Table 6).

4. Conclusion

The degradation profile of pentoxifylline was studied under various stress conditions monitored by LC–MS. The application of NMR and high resolution mass spectrometric studies helped in faster and accurate characterization of this degradation product. Degradation product 3, a novel *gem*-dihydroperoxide, obtained during oxidative stress studies was an important discovery as it may throw some light on this product formation in formulations or humans, its consequences and could be the subject of future research. In this study, we successfully used sub 2 μ reverse phase column technology, with UHPLC system for the development of efficient and robust method for the separation of closely related compounds. This method can be used to estimate the drug content in formulations and APIs. This fast UHPLC method increases throughput multiple folds, there by decreasing the cost of analysis and the time required for analysis.

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