



Original Article

Four new degradation products of doxorubicin: An application of forced degradation study and hyphenated chromatographic techniques[☆]Dheeraj Kaushik, Gulshan Bansal^{*}

Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala 147002, India

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ABSTRACT

Forced degradation study on doxorubicin (DOX) was carried out under hydrolytic condition in acidic, alkaline and neutral media at varied temperatures, as well as under peroxide, thermal and photolytic conditions in accordance with International Conference on Harmonization (ICH) guidelines Q1(R2). It was found extremely unstable to alkaline hydrolysis even at room temperature, unstable to acid hydrolysis at 80 °C, and to oxidation at room temperature. It degraded to four products (O-I–O-IV) in oxidative condition, and to single product (A-I) in acid hydrolytic condition. These products were resolved on a C₈ (150 mm × 4.6 mm, 5 μm) column with isocratic elution using mobile phase consisting of HCOONH₄ (10 mM, pH 2.5), acetonitrile and methanol (65:15:20, v/v/v). Liquid chromatography–photodiode array (LC–PDA) technique was used to ascertain the purity of the products noted in LC–UV chromatogram. For their characterization, a six stage mass fragmentation (MS⁶) pattern of DOX was outlined through mass spectral studies in positive mode of electrospray ionization (+ESI) as well as through accurate mass spectral data of DOX and the products generated through liquid chromatography–time of flight mass spectrometry (LC–MS–TOF) on degraded drug solutions. Based on it, O-I–O-IV were characterized as 3-hydroxy-9-desacetyldoxorubicin-9-hydroperoxide, 1-hydroxy-9-desacetyldoxorubicin-9-hydroperoxide, 9-desacetyldoxorubicin-9-hydroperoxide and 9-desacetyldoxorubicin, respectively, whereas A-I was characterized as deglucosaminyl doxorubicin. While A-I was found to be a pharmacopoeial impurity, all oxidative products were found to be new degradation impurities. The mechanisms and pathways of degradation of doxorubicin were outlined and discussed.

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1. Introduction

Drug regulatory agencies such as United States Food and Drug Administration (USFDA) and ICH have laid down very stringent guidelines for the control of impurities in drug substances and products [1–6]. ICH Q3A(R2) and Q3B(R2) guidelines specifically require the identification of impurities (process or degradation related) in any drug substance and product [3,4]. Identification of degradation related impurities (degradation products) remains a major challenge during product development. These arise due to chemical susceptibility of a drug molecule to varied chemical environments during product development, transportation and shelf life. Moreover, these are formed generally in minute amounts, which may not be sufficient to facilitate their characterization. Hence, ICH Q1A(R2) guidelines recommend forced degradation study (stress testing) on drug substance under different chemical

environments to facilitate isolation and/or characterization of all possible/major degradation products of the drug [2].

Anthracyclines constitute an important class of anti-tumor chemotherapeutic drugs with a broad spectrum of anticancer activity. DOX (also known as adriamycin) is a naturally anthracycline derivative and is a hydroxylated analog of daunorubicin (Fig. 1). It is particularly used for the treatment of disseminated neoplastic conditions such as acute lymphoblastic leukemia, acute myeloblastic leukemia, Wilm's tumor, neblastoma, soft tissue and bone sarcomas, breast carcinoma, ovarian carcinoma, transitional cell bladder carcinoma, thyroid carcinoma, Hodgkin's and non-Hodgkin's lymphomas, bronchogenic carcinoma and gastric carcinoma [7,8]. DOX is an important component of multi-chemotherapeutic drug regimen, and is usually given combined with cyclophosphamide, vincristine, bleomycin or prednisone. It is official in British Pharmacopoeia, United States Pharmacopoeia, Indian pharmacopoeia and Martindale [9–12]. The monograph about DOX in British Pharmacopoeia lists four impurities (Imp A–Imp D) [9] (Fig. 1). Some studies have reported on degradation and stability of DOX under varied conditions such as aqueous, photolytic and biological environments [13–20]. Of these, only Cielecka-Piontek et al. [13]

[☆]Peer review under responsibility of Xi'an Jiaotong University.^{*} Corresponding author. Fax: +91 175 2283073.E-mail addresses: gulshanbansal@rediffmail.com, gulshanbansal@gmail.com (G. Bansal).

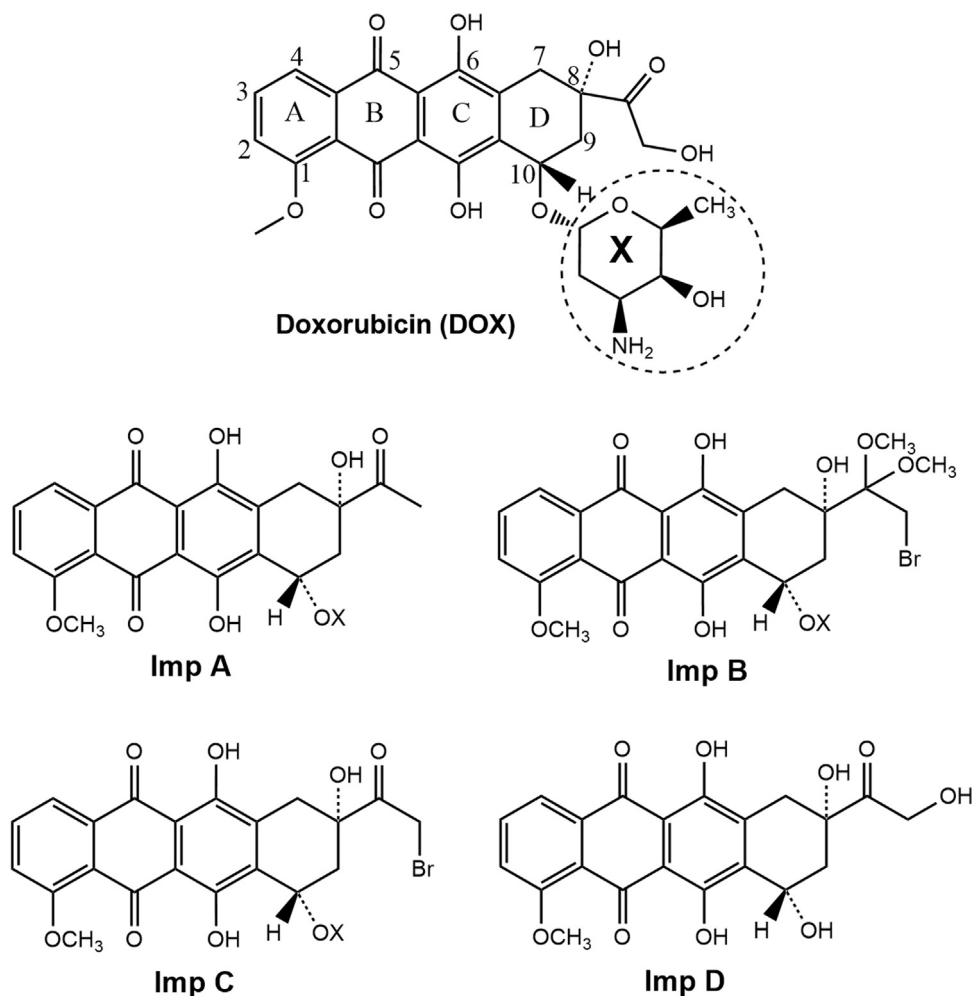


Fig. 1. Structure of doxorubicin (DOX) and its known impurities.

have proposed that on the basis of limited data, DOX and daunorubicin in solid state degrade to apolar degradation products that behave like 7-deoxyglycons. None of other studies has characterized the degradation products formed under different conditions. Herein, we report (i) a comprehensive forced degradation study on DOX under hydrolytic, photolytic, oxidative and thermal degradation conditions as prescribed in ICH Q1(R2) guidelines; (ii) structural characterization of degradation products through mass fragmentation and accurate mass spectral analyses; and (iii) most plausible mechanisms of DOX degradation.

2. Experimental

2.1. Drug and chemicals

Doxorubicin hydrochloride (DOX) was generously provided by Strides Arcolabs Pvt. Ltd. (Bangalore, India) as a gift sample. All analytical grade chemicals (sodium hydroxide, hydrochloric acid, hydrogen peroxide (30%) and ammonium formate) were supplied by Loba Chemical Pvt. Ltd. (Mumbai, India). The high performance liquid chromatography (HPLC) grade solvents and chemicals (methanol, acetonitrile and formic acid) were procured from Merck Specialist Pvt. Ltd. (Mumbai, India). HPLC grade water was obtained from Direct Ultra water purification system (Bio-Age Equipment and Services, SAS Nagar, India) in the laboratory.

2.2. Equipments

A high precision water bath and a hot air oven digitally controlling temperature variation of 1 and 2 °C, respectively (Narang Scientific Works, New Delhi, India) were employed for hydrolytic and thermal degradation studies. Photostability chamber which is equipped with digital controller capable of maintaining temperature and relative humidity (RH) within ± 2 °C and $\pm 5\%$, respectively (KBF 240, WTB Binder, Tuttlingen, Germany) was used for photodegradation studies. The light sources fixed in the chamber provided an illumination bank in compliance with ICH guideline Q1B [21]. Liquid chromatography–ultraviolet (LC–UV) analyses of the forced degradation samples were carried out on a binary HPLC system (515 pumps) equipped with a Rheodyne manual injector and a 2487 dual wavelength detector controlled by Empower 2 software (Waters, Milford, MA, USA). An Agilent C₈ (150 mm \times 4.6 mm, 5 μ m) column was used for chromatographic separation of the drug and degradation products. Purity of peaks of DOX and products in the degradation samples were ascertained by LC–PDA analysis using a binary HPLC system consisting of a 2707 auto-injector and a 2998 PDA detector (Waters, Milford, MA, USA). LTQ–XL ion trap quadrupole mass spectrometer (Thermo Scientific, Germany) was used to record multi-stage mass spectral (MSⁿ) data of DOX in positive mode of electrospray ionization (+ESI). For LC–MS–TOF analyses of DOX and degradation products, an Agilent 1100 series LC system (Agilent Technologies Inc., CA, USA) controlled by Hystar (Ver.3.1) software coupled with a microTOF–Q11 mass spectrometer (Bruker Daltonics GmbH, Germany) controlled

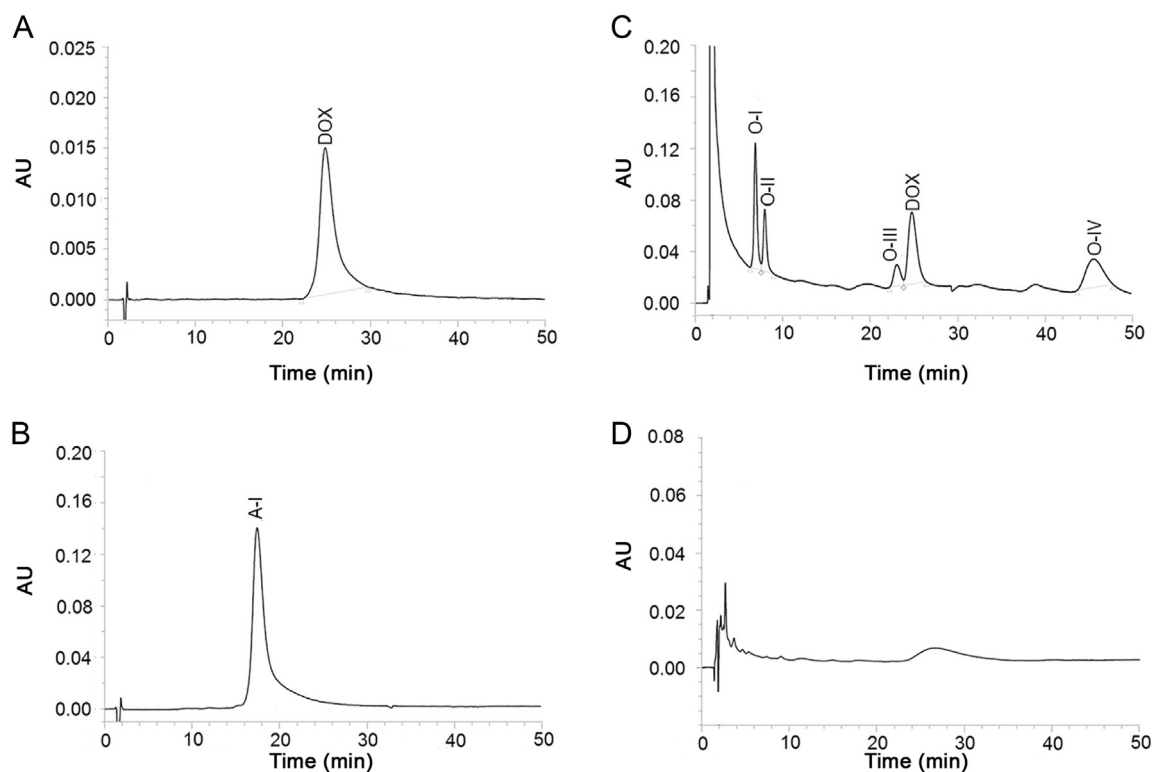


Fig. 2. Chromatogram of standard solution of DOX (A), and DOX solutions exposed to 0.1 M HCl at 80 °C (B), 30% H₂O₂ at room temperature (C), and 0.1 M NaOH at 80 °C (D).

Table 1
Clog P values and peak purity data of DOX and its degradation products.

Analyte	Clog P	Purity angle	Purity threshold
DOX	-0.4614	0.972	2.201
O-I	-1.7432	0.629	2.107
O-II	-1.0337	0.783	3.095
O-III	-0.8857	0.861	2.981
O-IV	1.5753	0.648	1.732
A-I	0.0695	1.029	2.985

by microTOF control software ver. 2.0 was used. A splitter allowing entry of only 35% of the eluent was placed before the mass detector in order to avoid signal saturation and detector contamination.

2.3. Forced degradation

DOX was exposed to conditions of hydrolysis, oxidation, dry heat and photolysis. Concentration of DOX in solution/suspension for the study was 0.1% (m/v). Solutions/suspensions of DOX in water, NaOH (0.1 M) and HCl (0.1 M) were exposed to 80 °C for a period of 8 h for hydrolytic degradation. Suspension of DOX in H₂O₂ (30%) was placed at 30 ± 5 °C in dark for 24 h for oxidative degradation. DOX in solid state was sealed in amber color vials and placed in hot air oven maintained at 50 °C for 30 days for thermal degradation. For photolytic degradation, 2 mL of a solution of DOX in acetonitrile was mixed with 3 mL of each stressor separately (*i.e.* 0.1 M HCl, 0.1 M NaOH and water) in transparent glass vials. These vials as well as the solid drug spreading as a thin layer in a petri dish were placed in the photostability chamber for 14 days to expose the samples to about 200 W h/m² of UV light and 1.2 million lx h of white light. Another similar set of solid drug and drug solutions wrapped well in aluminum foil was also placed in

the photostability chamber for the same period of time and served as dark control. Samples from each degradation condition were stored at 4 °C till analysis.

2.4. LC–UV and LC–PDA analyses

For chromatographic analysis, the thermal and photodegraded DOX in solid state was solubilized in methanol to produce solutions of concentration 0.1% (m/v). Each of these solutions as well as drug solutions/suspensions exposed to hydrolytic, oxidative and photolytic degradation conditions was diluted (1 in 10) with mobile phase. The drug suspension, wherever formed, was stirred well and sonicated for 5 min to make a homogenous suspension, whereas the acid and alkali degraded drug solutions were neutralized before dilution. Each diluted sample was filtered through a nylon membrane (0.45 μm, 13 mm) before injected on the HPLC system. Blank solution corresponding to each sample was also processed similar to the sample and its LC–UV analysis precedes that of the sample. Chromatographic separation of DOX and its degradation products was achieved by running 20 μL of degraded drug solution on a C₈ column (150 mm × 4.6 mm, 5 μm, Agilent) by isocratic elution with mobile phase composed of ammonium formate (10 mM, pH 2.5), acetonitrile and methanol (65:15:20, v/v/v) at a flow rate of 1 mL/min at ambient temperature (27 ± 2 °C). The eluent was detected at 234 nm. The purity of DOX and all its degradation products peaks in LC–UV chromatograms were established through LC–PDA analysis.

2.5. +ESI–MSⁿ and LC–MS–TOF studies

Six stage mass spectra of DOX in +ESI mode were recorded (+ESI–MS⁶) using ionization potentials increasing from 18.0 V to 28.0 V, and rationally selecting the precursor ion for each stage. Operating parameters of mass detector such as end plate offset

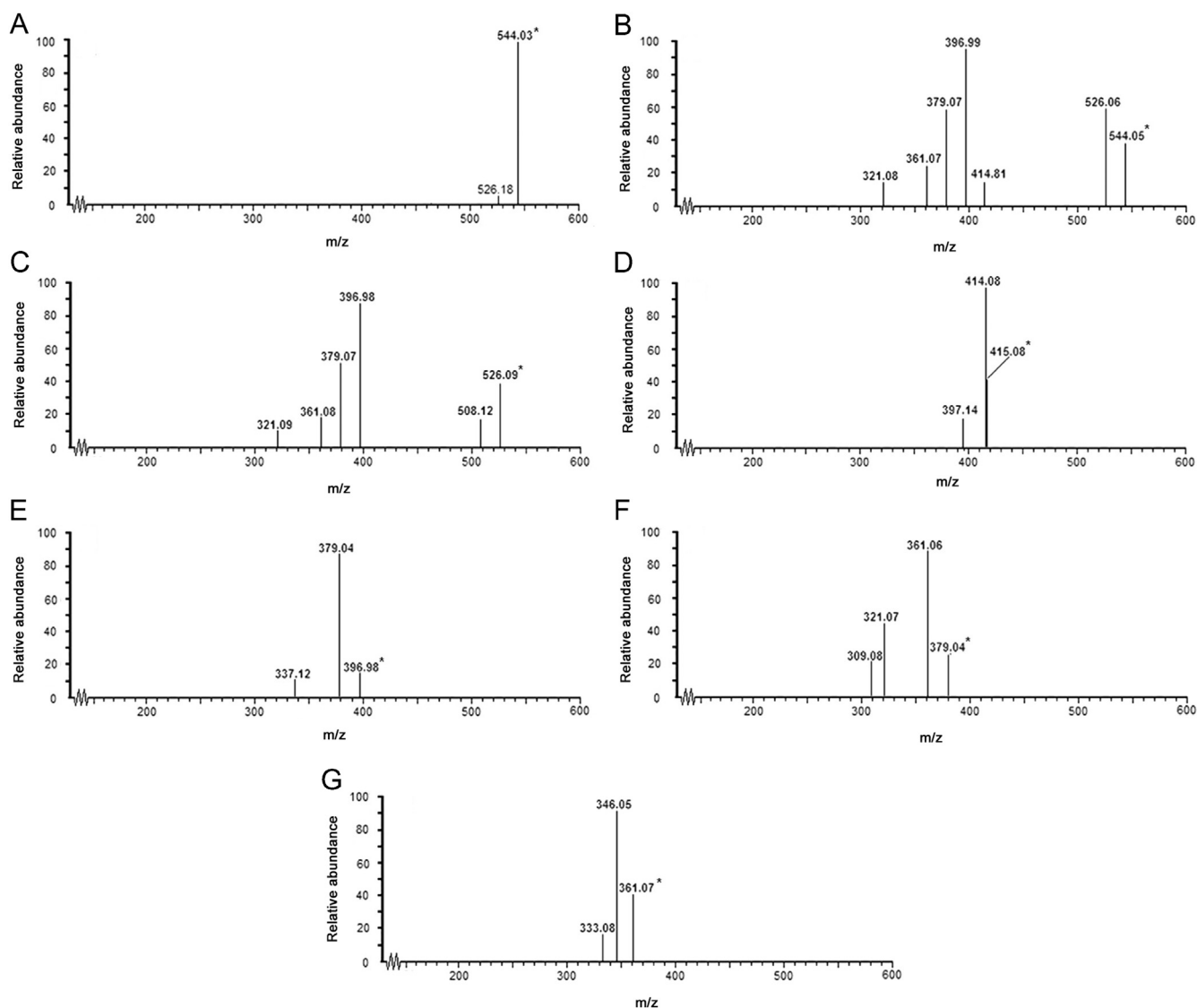


Fig. 3. Six stage mass fragmentation spectra of DOX. The precursor ion targeted to record each mass spectrum is marked by “*”.

Table 2

Precursor/parent ions and product ions in MS⁶ studies.

MS ⁿ stage	Precursor ion (m/z)	Product ions (m/z)
MS ¹	544 [M+H ⁺]	100%
MS ²	544	526, 415, 397 , 379, 361, 321
MS ^{3a}	526	508, 397 , 379, 361, 321
MS ^{3b}	415	414, 397
MS ⁴	397	379, 337
MS ⁵	379	361, 321, 309
MS ⁶	361	346, 333

voltage, capillary voltage, collision cell RF, nebulizer, dry gas flow, and dry temperature for recording MS⁶ spectra of DOX as well as LC–MS–TOF spectra of DOX and degradation products were optimized as –500 V, 4500 V, 400.0 vpp, 1.2 bar, 6.0 L/min and 200 °C, respectively. The LC–MS–TOF analyses were carried out by using ionization potentials of 10 and 15 V. All MS and TOF spectra were recorded over a mass range of 50–1000. Masses of various ion peaks in TOF spectra were recorded up to the fourth decimal precision for accurate mass measurements.

3. Results and discussion

3.1. Forced degradation behavior

DOX was detected as a sharp peak at 25.15 min wherein no impurity was noted in LC–UV chromatogram of standard solution (Fig. 2A). It degraded to a single product (A-I) under acid hydrolytic condition after 8 h (Fig. 2B), and four products (O-I–O-IV) under oxidative condition over 24 h (Fig. 2C). LC–PDA studies on acid hydrolyzed and oxidized drug solutions showed that purity angles of the peaks of DOX, O-I, O-II, O-III and O-IV and A-I were less than their purity threshold (Table 1), which indicated that all peaks were pure and no other products co-eluted with any of these peaks. Exposure of the drug to hydrolysis in water at 80 °C for 8 h, and to thermal degradation condition for 30 days produced no degradation products. In contrast, its exposure to 0.1 M NaOH at 80 °C for 8 h caused its almost total degradation (Fig. 2D). Even in mild alkaline medium (0.01 M NaOH) at 40 °C (Supplementary Fig. S2A) as well as at room temperature within 30 min, the drug degraded almost completely to several degradation products. In all alkali degradation conditions, the products were eluted as

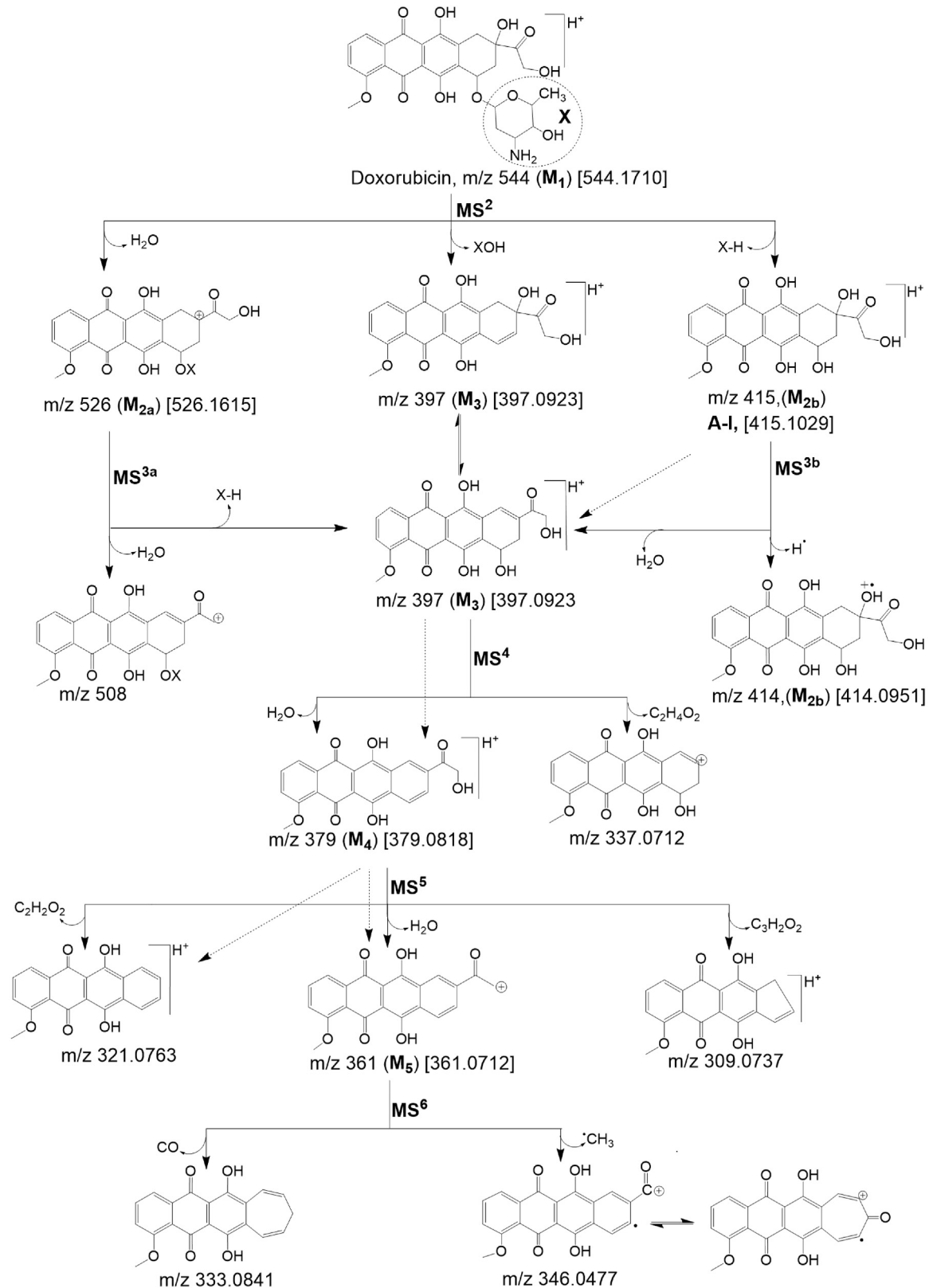


Fig. 4. Mass fragmentation pattern of DOX and its acid degradation product (A-I). The dotted arrows outline the fragmentation pathways of A-I.

complex bunch of peaks within 10–15 min that could not be resolved even after numerous modifications under chromatographic conditions such as composition of mobile phase, pH of buffer and stationary phase. Four LC–UV chromatograms showing the best, but still unacceptable resolution are given in [Supplementary Fig.](#)

S1. The degradation behavior of DOX under acidic and alkaline hydrolytic, and oxidative conditions was in consonant with the findings by other laboratories [13–18]. Comparison of the LC–UV chromatograms of the drug exposed to photolytic conditions ([Supplementary Fig. S2B and D](#)) with those of the drug stored in

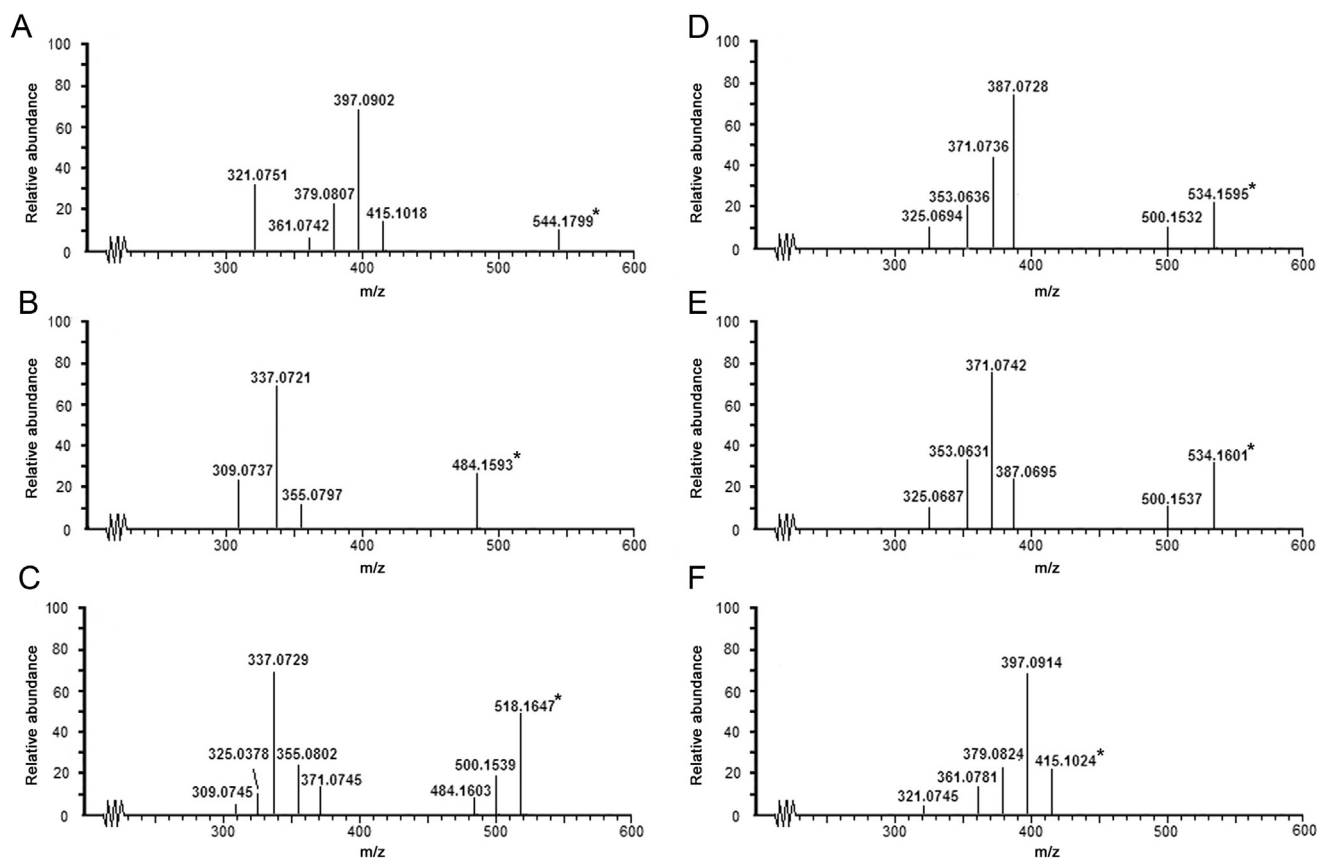


Fig. 5. LC-MS-TOF spectra of DOX and its degradation products (* indicates the parent ion).

dark (Supplementary Fig. S2C and E) in acidic and alkaline media, respectively revealed that no peak was formed due to photodegradation. Also, no additional photodegradation product was formed when solid drug was exposed to the photolytic conditions (Supplementary Fig. S2F). It indicates that degradation pattern of DOX under photolytic conditions in acidic and alkaline media is similar to that under hydrolytic condition, and hence the drug is suggested to be photostable.

However, these results of photostability of DOX are found conflicting, at the outset, with those in available reports on photodegradation of DOX. Wood et al. [15,16] have conducted a photodegradation study on DOX at different pH and in different containers. They found that (i) DOX degraded due to cleavage of glycosidic linkage in different media under photolytic conditions; (ii) both the glass and polyethylene container adsorb DOX; and (iii) the rate of drug degradation was inversely proportional to its concentration (i.e. 10% degradation in 100 $\mu\text{g}/\text{mL}$ solution of DOX and > 60% degradation in 10 $\mu\text{g}/\text{mL}$ solution after 168 h exposure to visible light). Nonetheless, the first two interpretations may not be considered really true with reference to our explanations that are as follows: Firstly, the glycosidic linkage is known to cleave under mild acid hydrolytic as well as strong alkaline hydrolytic conditions. However, there is no documented proof of its photolytic cleavage. Therefore, DOX might have degraded due to hydrolysis but not photolysis. Secondly, the major decrease in content of DOX in the degraded solution may also be, most likely, due to adsorption of DOX on the sample containers in addition to the actual degradation. The third finding actually acts as a support to photostability of DOX in the present study because herein we have subjected DOX to different degradation conditions using a solution of concentration 1000 $\mu\text{g}/\text{mL}$, which, according to Wood et al. [15,16] degrades at the least rate. In another study, DOX was found to degrade to 3-methoxysalicylic acid and an unstable

dihydroquinone after exposure to laser beam (λ 483 nm) for 48 h. These two products react with oxygen available in reaction mixture and form DOX and H_2O_2 . Hence, there was no net loss/degradation of DOX [15]. Other photodegradation studies have revealed that DOX degrades after exposure to long UV light in the presence of photosensitizers such as riboflavin. This riboflavin mediated photooxidation of DOX is further increased in the presence of histidine and urocanic acid [17–19]. The results of these literature reports suggest that the photodegradation of DOX is either reversible or is mediated by a photosensitizer. In the present study, the degradation of DOX was carried out using a drug concentration of 1000 $\mu\text{g}/\text{mL}$ without any photosensitizer. Hence, the drug does not degrade, and is termed as photostable under the tested conditions. However, in general, as light exposure tends to accelerate most chemical reactions, the drug may be recommended to be stored in containers affording protection from light. In addition, the drug is found stable at neutral pH, but its stability decreases significantly with increasing or decreasing the pH beyond 7.

3.2. Mass fragmentation pattern of DOX

Sleno et al. [22] have reported the mass fragmentation pattern of DOX on the basis of MS^3 spectra. In the present study, six stage mass fragmentation spectra (MS^6) of DOX were recorded (Fig. 3) for assisting the characterization of degradation products. The drug was detected at m/z 544 as parent ion (M_1) in MS^1 (Fig. 3A) corresponding to its molecular mass of 543 Da. The various product ions formed from M_1 and different precursor ions in all six stages are summarized in Table 2. Based on the comparison of product ions formed from different precursor ions in all mass spectra, a systematic mass fragmentation pattern of DOX was outlined (Fig. 4). The product ions formed directly from the

Table 3
Molecular formulae corresponding to the measured mass of various peaks in MS–TOF spectra of DOX and products O-IV, O-III and A-I.

Analyte peak	Observed mass (Da)	Mass difference	Most probable composition (Theoretical mass; Tolerance)*	
			For the mass difference	For the observed mass
DOX	544.1799			$C_{27}H_{30}NO_{11}^+$ (544.1813; –1.4)
	[M+H]⁺	129.0781	$C_6H_{11}NO_2^+$ (129.0790; –0.3)	
	415.1018	147.0897	$C_6H_{13}NO_3$ (147.0895; 0.7)	$C_{21}H_{19}O_9^+$ (415.1024; –0.6)
	397.0902	18.0116	H_2O (18.0100; 1.6)	$C_{21}H_{17}O_8^+$ (397.0918; –1.6)
	379.0807	18.0065	H_2O (18.0100; –4.1)	$C_{21}H_{15}O_7^+$ (379.0812; –0.5)
	361.0742	58.0056	$C_2H_2O_2$ (58.0055; 0.1)	$C_{21}H_{13}O_6^+$ (361.0706; 3.5)
	321.0751			$C_{19}H_{13}O_5$ (321.0763; –0.7) $C_{25}H_{26}NO_9^+$ (484.1602; –0.9)
O-IV	484.1593			
	[M+H]⁺	129.0796	$C_6H_{11}NO_2$ (129.0784; 1.1)	$C_{19}H_{15}O_7^+$ (355.0812; –1.5)
	355.0797	147.0872	$C_6H_{13}NO_3$ (147.0895; –1.8)	$C_{19}H_{13}O_6^+$ (337.0707; 1.4)
	337.0721	27.9984	CO (27.9949; 3.4)	$C_{18}H_{13}O_5^+$ (309.0758; –2.5)
O-IV	484.1593			
		34.0054	H_2O_2 (34.0055; –0.09)	$C_{25}H_{28}NO_{11}^+$ (518.1657; –0.9)
O-III	518.1647			
	[M+H]⁺	34.0044	H_2O_2 (34.0055; –1.07)	$C_{25}H_{26}NO_9^+$ (484.1602; 0.09)
	484.1603	129.0801	$C_6H_{11}NO_2$ (129.0790; 1.1)	$C_{19}H_{15}O_7^+$ (355.0812; –1.0)
	355.0802	147.084	$C_6H_{13}NO_3$ (147.0895; –5.5)	$C_{19}H_{13}O_6^+$ (337.0707; 2.2)
	337.0729	27.9984	CO (27.9949; 3.5)	$C_{18}H_{13}O_5^+$ (309.0758; –1.3)
	309.0745			
DOX	544.1799			
		129.0775	$C_6H_{11}NO_2$ (129.0789; –1.5)	$C_{21}H_{19}O_9^+$ (415.1024; 0.04)
A-I	415.1024			
	[M+H]⁺	18.0110	H_2O (18.0105; 0.4)	$C_{21}H_{17}O_8^+$ (397.0918; –0.4)
	397.0914	18.0090	H_2O (18.0105; +0.6)	$C_{21}H_{15}O_7^+$ (379.0812; 1.2)
	379.0824	18.0043	H_2O (18.0105; –1.6)	$C_{21}H_{13}O_6^+$ (361.0706; 7.4)
	361.0781	58.0079	$C_2H_2O_2$ (58.0049; 3.0)	$C_{19}H_{13}O_5$ (321.0763; –1.8)
	321.0745			

respective precursor ion at each stage are marked as bold (Table 2) and subsequently, M_1 fragmented directly to product ions of m/z 526, 415 and 397 (Fig. 3B) by the loss of water and amino sugar moiety as specified in Fig. 4. The heaviest fragment m/z 526 (M_{2a}) was used as precursor ion to record MS^{3a} spectrum wherein it fragmented to product ions of m/z 508 by the loss of water and m/z 397 by the loss of amino sugar moiety (Fig. 3C). The fragment of m/z 415 (M_{2b}) fragmented to m/z 414 and 397 in MS^{3b} through the loss of a hydrogen radical and amino sugar moiety, respectively (Fig. 3D). Incidentally, product ion of m/z 397 was formed in MS^2 , MS^{3a} as well as MS^{3b} . Hence, it was taken as precursor ion to record subsequent mass spectra. Fragmentation of m/z 397 (M_3) produced product ions of m/z 379 (M_4) and 337 in MS^4 spectrum due to the loss of water molecule and a $C_2H_4O_2$ respectively from side chain of ring A (Fig. 3E). The heaviest fragment of m/z 379 (M_4) in MS^4 spectrum was further fragmented to product ions of m/z 361 (M_5), 321 and 309 (Fig. 3F). Finally, M_5 was employed as precursor ion to generate MS^6 spectrum wherein it fragmented to product ions of m/z 333 by the loss of CO moiety [22] and m/z 346 by the loss of a methyl radical (Fig. 3G). In addition to MS^6 study, DOX was also analyzed through LC–MS–TOF to generate accurate mass spectral data. The TOF spectrum of DOX (Fig. 5A) showed parent ion at m/z 544.1799. The major fragments were noted at m/z 526.1619, 415.1024, 397.0902, 379.0807, 361.0742 and 321.0751. These masses were found to be very close to the theoretical accurate masses and the structures of product ion of m/z 526, 415, 397, 379, 361 and 321 (Table 3) formed during MS^6 study which supported the proposed structural assignment to these ions.

3.3. LC–MS–TOF studies for characterization of degradation products

Structures of the degradation products were characterized with the help of MS^6 fragmentation pattern of DOX, comparative accurate mass spectral data of the drug and products generated through LC–MS–TOF analysis of degraded drug solutions, and Elemental Composition Calculator Software that provided the most probable molecular formula corresponding to ion peaks in the mass spectra. The four oxidative products (O-I–O-IV) and the single acid hydrolyzed product (A-I) detected in LC–UV chromatogram were detected in total ion chromatogram (TIC). The LC–MS–TOF spectra of DOX and each product are given in Fig. 5 and their accurate mass spectral data are summarized in Table 3 and 4.

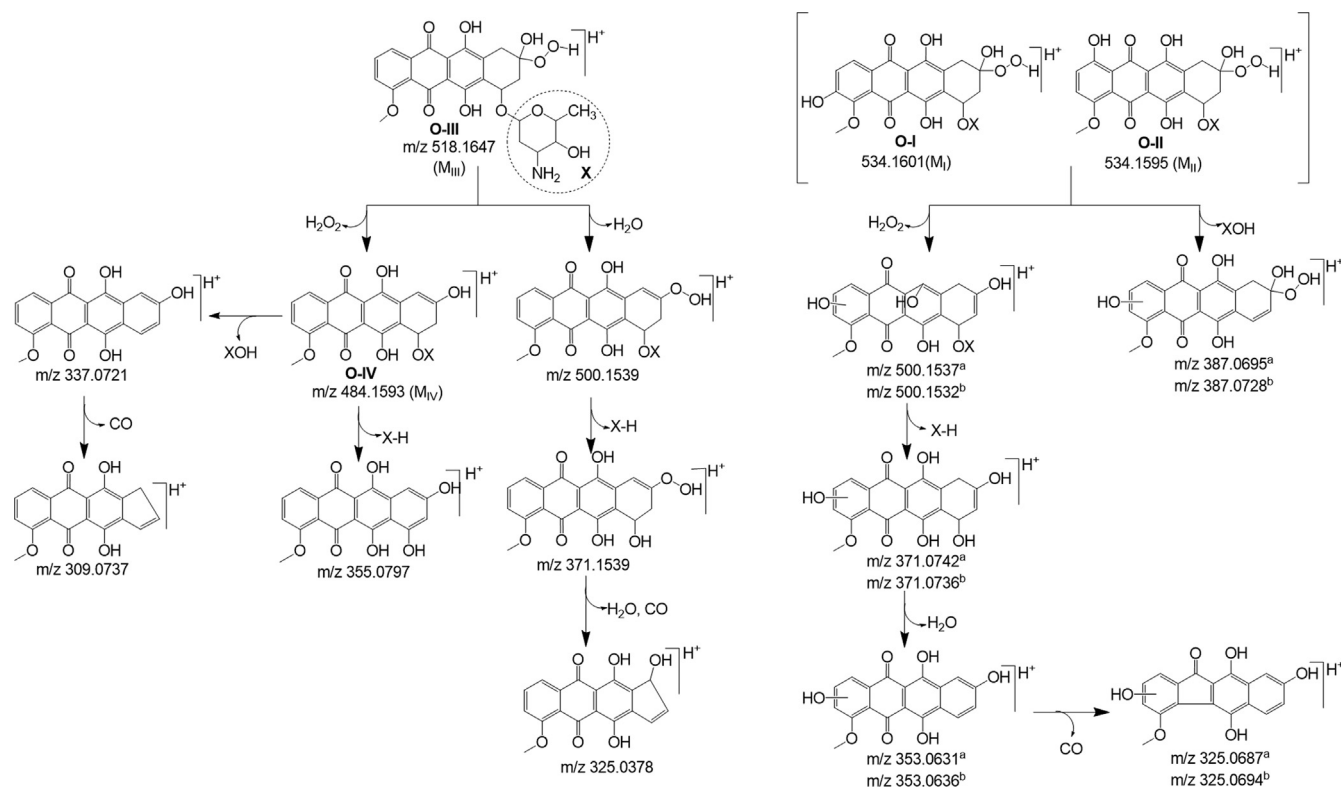
3.3.1. Product O-IV

The heaviest peak in MS–TOF spectrum of O-IV was noted at m/z 484.1593 (Fig. 5B) which was assigned as its parent ion (M_{IV}) peak. An even molecular mass of M_{IV} suggested an odd number nitrogen atom [23]. Hence, the single nitrogen atom in amino sugar component of DOX was intact in O-IV. The mass difference of 60.0226 Da between M_1 and M_{IV} was found to correspond to molecular formula $C_2H_4O_2$ (60.0231 Da) [24]. The possible molecular structures corresponding to $C_2H_4O_2$ include acetic acid, 2-hydroxyacetaldehyde (OHCH₂CHO) and 1,2-ethylenediol. Out of these, 2-hydroxyacetaldehyde was possible to form from hydroxylacetyl side chain on ring D of DOX. Hence, O-IV was proposed to form by the loss of COCH₂OH group from DOX that was possible through Baeyer Villiger oxidation in the presence of H_2O_2 . Based on this proposition, O-IV was suggested to be

Table 4

Molecular formulae corresponding to the measured mass of various peaks in MS–QTOF spectra of products O-I and O-II.

Analyte peak	Observed mass (Da)	Mass difference	Most probable composition (Theoretical mass; Tolerance)*	
			For the mass difference	For the observed mass
O-III	518.1647			
		15.9948	O (15.9949; -0.1)	
O-II	534.1595			$C_{25}H_{28}NO_{12}^+$ (534.1606; -1.1)
		34.0063	H_2O_2 (34.0055; 0.8)	$C_{25}H_{26}NO_{10}^+$ (500.1551; -1.9)
	500.1532			
		147.0867	$C_6H_{13}NO_3$ (147.0895; -2.8)	$C_{19}H_{15}O_9^+$ (387.0711; 1.7)
	387.0728			
		129.0796	$C_6H_{11}NO_2$ (129.0790; 0.6)	$C_{19}H_{15}O_8^+$ (371.0761; -2.5)
	371.0736			
		18.0100	H_2O (18.0106; -0.6)	$C_{19}H_{13}O_7^+$ (353.0656; -1.9)
	353.0636			
		27.9942	CO (27.9949; -0.7)	$C_{18}H_{13}O_6^+$ (325.0707; -1.3)
	325.0694			
O-III	518.1647			
		15.9954	O (15.9949; 0.5)	
O-I	534.1601			$C_{25}H_{28}NO_{12}^+$ (534.1606; -0.5)
		34.0064	H_2O_2 (34.0055; -1.8)	$C_{25}H_{26}NO_{10}^+$ (500.1551; -1.4)
	500.1537			
		147.0906	$C_6H_{13}NO_3$ (147.0895; 1.0)	$C_{19}H_{15}O_9^+$ (387.0711; -1.6)
	387.0695			
		129.0795	$C_6H_{11}NO_2$ (129.0790; 0.5)	$C_{19}H_{15}O_8^+$ (371.0761; -1.9)
	371.0742			
		18.0111	H_2O (18.0106; 0.5)	$C_{19}H_{13}O_7^+$ (353.0656; -2.5)
	353.0631			
		27.9944	CO (27.9949; -0.5)	$C_{18}H_{13}O_6^+$ (325.0707; -2.0)
	325.0687			

**Fig. 6.** Proposed mass fragmentation pattern of oxidative products (O-I–O-IV). The fragments superscripted with “a” belong to O-I while those with “b” belong to O-II.

9-desacetyldoxorubicin, which can exist in two forms due to keto-enol tautomerism. This proposition was supported by a similar kind of product under oxidative conditions reported to form from idarubicin [25]. The TOF spectrum of O-IV showed product ions at m/z 355.0797, 337.0721 and 309.0737. The fragment of m/z 355.0797 was 129.0796 Da less than M_{IV} and was proposed to form by the loss of amino sugar moiety from M_{IV} similarly as M_{2b} forming from M_1 and M_{2a} , respectively. The m/z values 337.0721

and 309.0737 matched very closely with accurate theoretical mass of structures assigned to fragments m/z 337 and 309, respectively, in MS^4 and MS^5 spectra of DOX. Based on this comparative mass spectral data, the suggested structure of O-IV was proposed to undergo fragmentation as shown in Fig. 6. The fragment of m/z 309.0737 was possible to form from m/z 337.0721 by the loss of CO group (27.9984 Da) similar to mass fragmentation of phenols or alcohols reported in standard literature [24].

3.3.2. Product O-III

It was detected as the heaviest and major peak at m/z 518.1647 (Fig. 5C) that was assigned as its parent ion (M_{III}) peak. An even molecular mass of M_{III} suggested an odd number of nitrogen atoms [23]. Hence, the single nitrogen atom present in amino sugar component of DOX was suggested to be intact in O-III, similarly as in O-IV. The MS-TOF spectrum also showed fragment ion of m/z 500.1539, 484.1603, 371.0745, 355.0802, 337.0729, 325.0378 and 309.0745. Of these, the fragments of m/z 484.1603, 355.0802, 337.0729 and 309.0745 were also noted in TOF spectrum of O-IV. It indicated that O-III may be a derivative of O-IV itself. M_{III} was 34.0044 Da heavier than m/z 484.1603, and this mass difference was found to correspond to an H_2O_2 molecule (34.0055 Da) [24]. Based on these results, it was suggested that O-III might be formed by the addition of H_2O_2 across the ketone group generated on ring D in keto form of O-IV. This contention was supported by an earlier study, in which a similar hydroperoxide degradation product was proposed to form from idarubicin [25]. Hence, O-III was proposed to be 9-desacetyl-doxorubicin-9-hydroperoxide, which underwent mass fragmentation (Fig. 6) in consonant with its MS-TOF spectrum. The fragment of m/z 484.1603 was possible to form by the loss of H_2O_2 from M_{III} , whereas the product ions of m/z 355.0802, 337.0729 and 309.0745 were proposed to form

similarly as from M_{IV} . The product ions of m/z 500.1539 and 371.0745 were possible to form by the loss of H_2O molecule followed by the loss of X-H. The fragment of m/z 325.0387 was proposed to form from m/z 371.0745 through one or two steps.

3.3.3. Products O-II and O-I

The LC-MS-TOF spectra of O-II and O-I showed major and heaviest peaks at m/z 534.1595 and 534.1601, hence they were assigned as parent ions (M_{II} and M_I , respectively) (Fig. 5D and E). An even mass of M_I and M_{II} suggested an odd number of nitrogen atoms in the products [23], which indicated the single nitrogen atom in the amino sugar component in DOX to be intact in both O-I and O-II. The very similar values of M_I and M_{II} (Tolerance of 0.7% as per Elemental Composition Calculator Software) indicated that both O-I and O-II have the same chemical formula. However, O-I and O-II eluted at different retention times *i.e.* 6.9 min and 7.8 min, respectively (Fig. 2), which suggested these products have different structures. The TOF spectra of both products displayed the same fragment ions (Fig. 5D and E). This comparative information on chromatographic and mass spectral data of O-I and O-II suggested that though they have similar chemical formula, they have different structures, which may be isomers. The TOF spectrum of O-II showed fragment ions of m/z 500.1532, 387.0728, 371.0736,

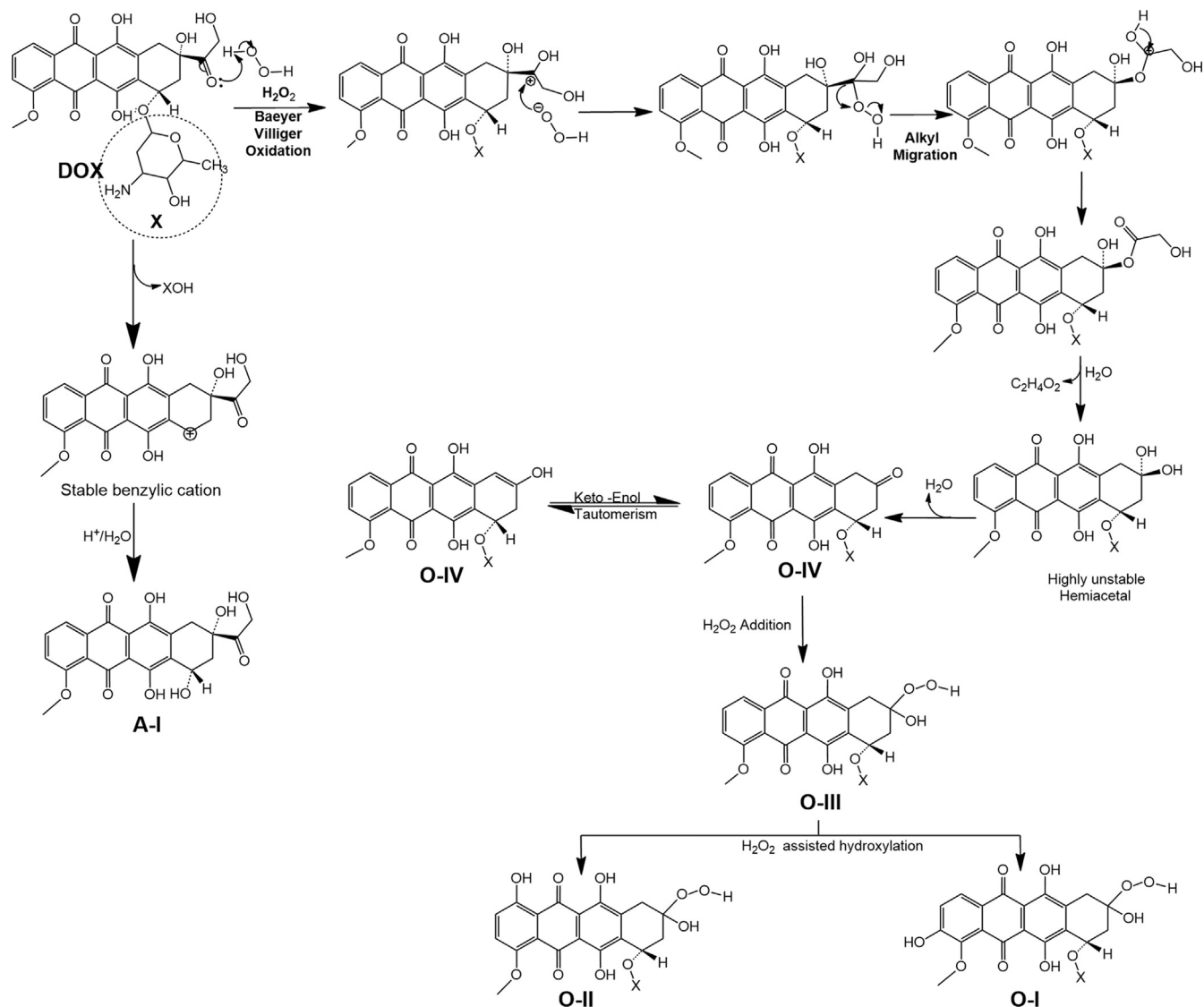


Fig. 7. Mechanism of degradation of DOX.

353.0636 and 325.0694 (Fig. 5D). The heaviest fragment (m/z 500.1532) was 34.0063 Da less than that of M_{II} , and this mass difference was found to correspond to an H_2O_2 molecule (34.0055 Da). The same fragment was also noted as the heaviest fragment in TOF spectrum of O-III, wherein it was proposed to form by the loss of a water molecule (Fig. 6). Moreover, M_{III} was also found to fragment to m/z 484.1603 (M_{IV}) by the loss of an H_2O_2 molecule (Fig. 6). These comparisons in mass spectral data indicated that hydroxyhydroperoxide moiety is present in M_{II} , similarly as in M_{III} , which implied that O-II is a derivative of O-III itself. Further, the mass difference of 15.9958 Da between M_{III} and M_{II} was found to correspond to an oxygen atom (15.9949 Da). It suggested that O-II might form by the addition of an oxygen atom in M_{III} under the oxidative condition, which was possible through peroxide catalyzed oxidation of ring A leading to the formation of a hydroxylated (phenol) derivative. Further, this hydroxylation in ring A could occur at position *ortho* or *para* with respect to methoxy group (activating group). A logical co-relation among chromatographic elution data and comparative mass spectral data of O-I and O-II, and possibility of hydroxylation in ring A of DOX suggested that O-I and O-II might be positional isomers (*ortho* and *para*) of the hydroxylated derivative of O-III. As O-II eluted after O-I (Fig. 2), O-I might be more polar than O-II. This co-relation between polarity and elution order was supported by the observation that elution order of O-III, O-IV and DOX was in concordance with their lipophilicity, determined as *Clog P* by ChemBioDraw Ultra 12.0 software (Table 1). The possible *ortho* hydroxyl derivative of O-III was predicted to be more polar than the *para* isomer (Table 1). Hence, O-I was proposed to be an *ortho* isomer (*o*-anisole derivative) whereas O-II to be the *para* isomer (*p*-anisole derivative), which underwent mass fragmentation in agreement with their mass spectral data (Fig. 6). Hence, O-I and O-II were suggested to be 3-hydroxy-9-desacetyl-doxorubicin-9-hydroperoxide and 1-hydroxy-9-desacetyl-doxorubicin-9-hydroperoxide, respectively.

3.3.4. Product A-I

It was detected as the heaviest ion peak at m/z 415.1024 (Fig. 5F), which was assigned as its parent ion (M_A) peak. An odd molecular mass of M_A indicated an even number nitrogen or no nitrogen atom [23], which suggested that amino sugar moiety might be absent in A-I. M_A was also noted as a product ion in MS^2 spectrum of DOX, wherein it was proposed to form by the loss of amino sugar moiety (X-H) from the parent ion of DOX. Based on this, A-I was proposed to be deglucosaminyl doxorubicin formed by the loss of the glucosamine moiety due to cleavage of glycosidic linkage between the tetracycline ring and the glucosamine moiety. Mass fragmentation of the proposed A-I to product ions of m/z 397.0914, 379.0824, 361.0781 and 321.0745 (Fig. 4) was in agreement with its TOF spectrum.

3.4. Drug degradation mechanisms

Degradation of DOX to deglucosaminyl doxorubicin (A-I) under acid hydrolytic condition was possible through well known acid catalyzed cleavage of glycosidic linkage (Fig. 7) similar as the reports on the degradation of other similar drug molecules such as idarubicin, doxorubicin, 4'-deoxydoxorubicin, 4'-O-methyl doxorubicin, 4'-epidoxorubicin, doxorubinol, daunorubicin and carminomycin under acidic conditions [14,25]. Degradation of DOX to O-IV (9-desacetyl-doxorubicin) and then to O-III (9-desacetyl-doxorubicin-9-hydroperoxide) was possible through Baeyer Villiger oxidative deacetylation following the addition of H_2O_2 (Fig. 7), similar to the oxidative degradation of idarubicin [25]. The products O-I (3-hydroxy-9-desacetyl-doxorubicin-9-hydroperoxide) and O-II (1-hydroxy-9-desacetyl-doxorubicin-9-hydroperoxide) were proposed to form by peroxide assisted hydroxylation in ring A of doxorubicin. This proposition was supported by a report that disclosed and discussed the formation of alkoxy and aryloxy substituted phenols

from the corresponding alkoxy and aryloxy benzene [26]. However, a comparison of the results of the present study with degradation behavior of idarubicin reported earlier by our laboratory [25] revealed that such hydroxylated products did not form from idarubicin. This difference in degradation behaviors of doxorubicin and idarubicin was attributed to the methoxy group in ring A in doxorubicin, which is a moderate activating group and directs substitution at *ortho* and *para* position [27].

4. Conclusion

DOX was subjected to forced degradation under the conditions of hydrolysis (water, acid and alkali), peroxide oxidation, dry heat and photolysis as recommended in ICH guidelines Q1(R2). DOX degraded to several degradation products in alkaline medium even at room temperature that could not be resolved. Four degradation products (O-I–O-IV) were formed in oxidative conditions, and a single product (A-I) was formed in acid hydrolytic condition. They were resolved by a single isocratic LC–UV method and characterized by MS^6 and LC–MS–TOF studies as 3-hydroxy-9-desacetyl-doxorubicin-9-hydroperoxide (O-I), 1-hydroxy-9-desacetyl-doxorubicin-9-hydroperoxide (O-II), 9-desacetyl-doxorubicin-9-hydroperoxide (O-III), 9-desacetyl-doxorubicin (O-IV), and deglucosaminyl doxorubicin (A-I). While O-I–O-IV were found to be new degradation impurities, A-I was found to be a pharmacopoeial impurity of DOX. The drug remained stable to hydrolytic degradation in neutral medium, thermal degradation and photolytic degradation.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.jpha.2015.05.003>.

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