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Isolation and characterization of degradation impurities in epirubicin hydrochloride injection

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ABSTRACT

The degradation of epirubicin hydrochloride aqueous formulation has been investigated during stability study. Some unknown degradation impurities were detected and out of these, three were characterized. These degradation impurities were isolated, enriched and were subjected to mass and NMR spectral studies. Based on the spectral data these were characterized as epirubicin dimer (impurity-1), 4-(4-amino-5-hydroxy-6-methyl-tetrahydro-pyran-2-yloxy)-2,5,12-trihydroxy-7-methoxy-6,11-dioxo-1,2,3,4,6,11-hexahydro-naphthacene-2-carboxylic acid hydroxymethyl ester (impurity-3) and 4-(4-amino-5-hydroxy-6-methyl-tetrahydro-pyran-2-yloxy)-2,5,12-trihydroxy-7-methoxy-6,11-dioxo-1,2,3,4,6,11-hexahydro-naphthacene-2-carboxylic acid (impurity-4). Structure elucidations of these degradation impurities are discussed in detail. Out of these degradation impurities, epirubicin dimer (impurity-1) has been previously identified while the other two impurity-3 and impurity-4 were previously unreported.

1. Introduction

Epirubicin hydrochloride is an anthracycline cytostatic antibiotic with the widest antineoplastic spectrum, an analog of doxorubicin hydrochloride, differing only in position of the C-4′ hydroxy group of the sugar moiety [1]. Epirubicin is obtained by chemical transformation of a substance produced by certain strains of *Streptomyces peucetius*. The basic anthracycline structure of epirubicin consists of a tetracyclic quinoid moiety glycosidally linked to the aminosugar acosamine. Epirubicin hydrochloride is currently used as high effective antineoplastic drug for treatment of a variety of tumors. Epirubicin has a lower incidence of toxic side effects when compared with doxorubicin at comparable doses. At present, only one official monograph in pharmacopoeia for the quality control of epirubicin hydrochloride is available [2]. Some other methods are also available for analysis of epirubicin hydrochloride [3–6] all these methods are not suitable due to LC–MS incompatibility and separation point of view.

The present study describes the isolation and characterization of degradation impurities in epirubicin hydrochloride formulation. These impurities were isolated using preparative LC and characterized by using NMR and LC–MS/MS spectral data.

2. Materials and methods

2.1. Chemicals

The chemicals and reagents used for the analysis and purification purpose of epirubicin and degradation impurities:

Trifluroacetic acid (AR grade), Supplier: Real Chemsys, India;
Ortho-phosphoric acid (AR grade), Supplier: Ranbaxy Fine Chemicals Limited, India;
Acetonitrile (HPLC grade), Supplier: S.D. Fine Chem. Limited, India;
Methanol (HPLC grade), Supplier: S.D. Fine Chem. Limited, India;
Water: Highly pure Milli Q water was used with the help of Millipore Milli-Q plus purification system;

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Table 1
Analytical gradient program

Time (min)	Mobile phase A (%, v/v)	Mobile phase B (%, v/v)
0	70	30
40	60	40
45	20	80
50	20	80
51	70	30
60	70	30

Table 2
Preparative gradient program

Time (min)	Mobile phase A (%, v/v)	Mobile phase B (%, v/v)
0	100	0
50	85	15
60	80	20
65	20	80
85	20	80
86	100	0
95	100	0

Epirubicin hydrochloride injection, Source: Dabur Research Foundation, contains epirubicin hydrochloride 2 mg/mL, sodium chloride, CO_2 in water, HCl for pH adjustment (pH 3.0);
Epirubicin HCl and known impurities, Source: Synbias Pharma Limited, Ukraine.

2.2. Analytical conditions

The chromatographic separation was performed on Agilent HPLC system consisting of Agilent Technologies 1100 series quaternary solvent delivery module, UV detector and autosampler. The data were processed using software Chemstation (LC-3D rev. A.09.03 [1417]). The HPLC method was developed for the analysis of epirubicin hydrochloride injection and its degradation impurities. The analytical conditions used were, C18 column (Inertsil-ODS 3 V, 250 mm × 4.6 mm i.d., 5 μm particle size), column oven temperature 35 °C, with a flow rate of 1.0 mL/min, the gradient condition was developed using mobile phase A (0.1% trifluroacetic acid) and mobile phase B (mixture of acetonitrile, methanol, trifluroacetic acid in the ratio of 80:20:0.1) and the detection was performed at 254 nm. The impurities were eluted according to the step gradient by changing the % of mobile phase B at different times (Table 1). The samples were prepared in diluent (diluent—50:50 water, pH 2.5 with H_3PO_4 and acetonitrile). Final concentration of sample solution for HPLC injection was 1000 ppm. The HPLC chromatograms were integrated for the detected impurity peaks, while peaks arising from blank, diluent and placebo were not integrated in samples.

2.3. Isolation of impurities

Degradation impurities were isolated form epirubicin hydrochloride injection stability samples by using preparative LC. The preparative LC system used was a Shimadzu chromatograph equipped with LC-8A solvent delivery module, SCL-8A system controller, SIL-8A auto injector, SPD-6A UV–vis detector, FCV 100B fraction collector and data recorder C-R6A chromatopac. For the isolation, an Inertsil ODS-3 column with dimension of 250 mm × 20 mm, packed with 6 μm particle size was used. The solvent A is 0.1% trifluroacetic acid and solvent B is mixture of acetonitrile, methanol and trifluroacetic acid in the ratio of 80:20:0.1. The gradient programme was developed using mobile phase A (mixture of solvent A and solvent B in the ratio of 80:20) and mobile phase B (solvent B) with a flow rate of 20 mL/min and the detection was performed at isolation, about 1500 mL epirubicin hydrochloride injection (accelerated condition), 254 nm. The gradient program (Table 2) was used for isolation of impurities. For concentration 2 mg/mL, were used. Total 60 runs were performed using 20 mL (40 mg) in each loading on to the preparative LC column. The major peaks were isolated individually. All isolated fractions having maximum purity were pooled together for individual impurities. All isolated and final pooled fractions were analyzed by analytical HPLC. The solvent evaporation was performed under high vacuum using Buchi rotavapor R-124 equipped with vacuum system B-178 and water bath B-480. The concentrated fractions were lyophilized using freeze dryer (Instrument model-Virtis Genesis 25EL) and the lyophilized samples were used for identification purpose. Finally about 10 mg of each impurity of interest could be isolated in solid form.

2.4. LC–MS/MS analysis

Electrospray ionization and tandem mass spectrometry experiments were performed using triple quadrupole mass spectrometers namely Micromass Quattro Micro. (The positive electrospray data were obtained by switching the capillary voltage between +3.463 to −3.463 kV, respectively, cone voltage 26 V, source temperature 120 °C, desolvation temperature 150 °C, desolvation gas 500 L/min.

Table 3
Stability study of epirubicin HCl injectable solution in different condition

Time interval (month)	Impurity-1 (%)	Impurity-2 (%)	Impurity-3 (%)	Impurity-4 (%)
Epirubicin hydrochloride formulation stability study at 2–8 °C, solution pH 3.0				
Initial	0.17	Not detected	Not detected	Not detected
1	0.31	0.02	0.02	0.04
2	0.42	0.06	0.04	0.09
3	0.55	0.09	0.06	0.17
6	0.55	0.20	0.09	0.23
9	0.58	0.25	0.12	0.30
12	0.64	0.33	0.17	0.41
Epirubicin hydrochloride formulation stability study at 25 °C, solution pH 3.0				
Initial	0.17	Not detected	Not detected	Not detected
1	0.32	0.19	0.15	0.15
3	0.93	0.51	0.62	0.55
6	1.12	0.86	1.03	1.53

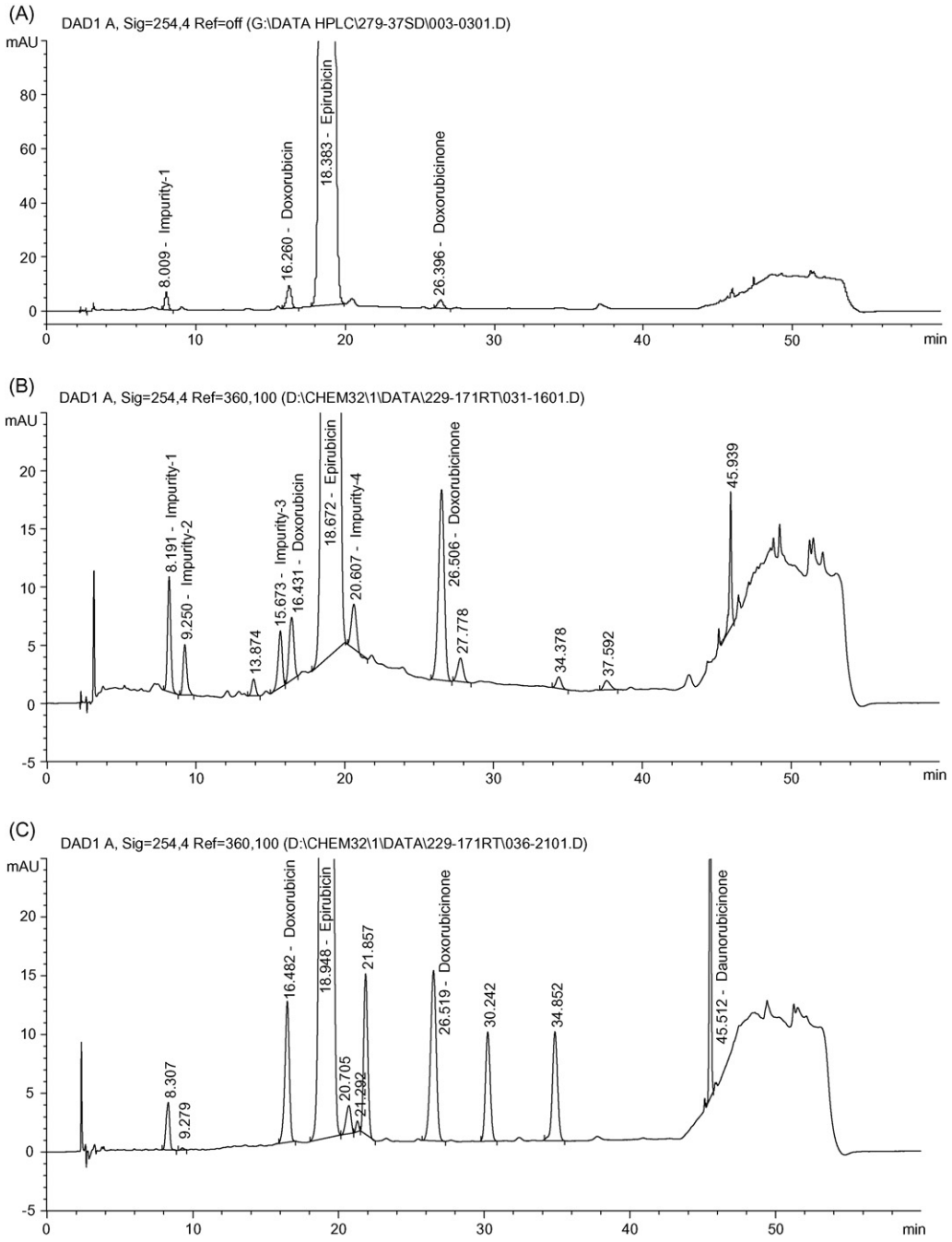


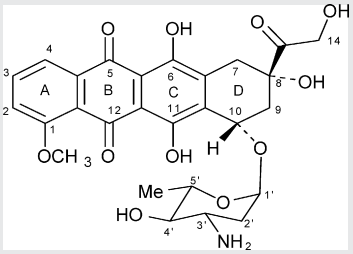
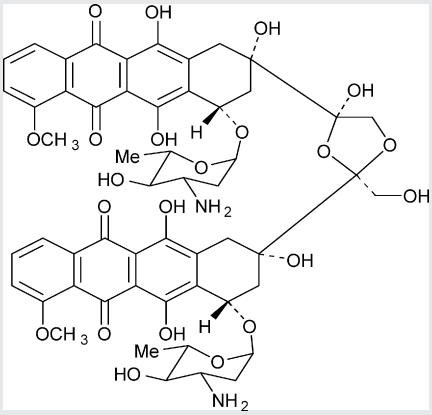
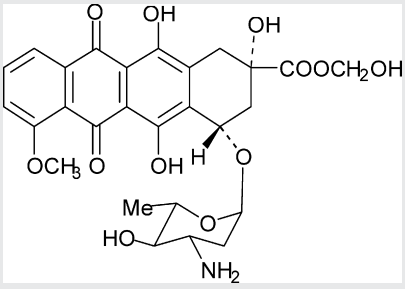
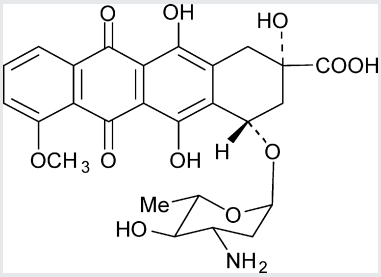
Fig. 1. Representative HPLC chromatograms of epirubicin hydrochloride injection. (A) Epirubicin injection: initial. (B) Epirubicin injection: 25 °C/60% RH, 3 months. (C) Epirubicin injection spiked with known impurities.

The MS–MS data were obtained using collision potential 20 V and nitrogen gas in collision cell.) The above conditions were used to perform the experiment on impurity-1 and impurity-2 for characterization. The MDS Sciex model API 4000 were used to characterization of impurity-3 and impurity-4. The positive electrospray data were obtained by switching the capillary voltage between +5500 and −4500 V, respectively. Collision potential (22 V) and nitrogen gas was used in the collision cell for MS–MS studies. The above (model API 4000) conditions were applied to perform the experiment on impurity-3 and impurity-4 for characterization. The samples were prepared in diluent (50:50 water and acetonitrile).

2.5. NMR spectroscopy

The NMR experiments were performed on Bruker spectrometers operating at 300 MHz using deuterated solvents DMSO-d_6, $CDCl_3$, D_2O and CF_3COOD (Instrument model: Bruker DPX 300 MHz). The ^1H chemical shift values were reported on the δ scale in ppm, relative to Tetra methyl silane ($\delta = 0.00$).

Table 4
Name, structure and *m*/*z* value of epirubicin and degradation impurities

Name	Structure	Molecular weight
Epirubicin		543
Impurity-1		1086
Impurity-3		559
Impurity-4		529

3. Results and discussion

The present study describes the isolation and characterization of degradation impurities formed during long-term storage of aqueous formulation at 2–8 °C as well as accelerated condition 25 °C/60% RH (Table 3). The representative HPLC chromatogram of epirubicin hydrochloride injection initial, stored and spiked impurities are shown in Fig. 1A–C. The epirubicin impurity-1, impurity-3 and impurity-4 were isolated and characterized. The structure of impurity-2 could not be confirmed. The structure and molecular weights of epirubicin and the identified impurities are given in Table 4. The detail discussions of all four impurities are given below.

3.1. Structure elucidation of epirubicin impurity-1

This impurity was isolated in pure solid form. The ESI mass spectrum of epirubicin impurity-1 (Fig. 2) gave protonated molecular

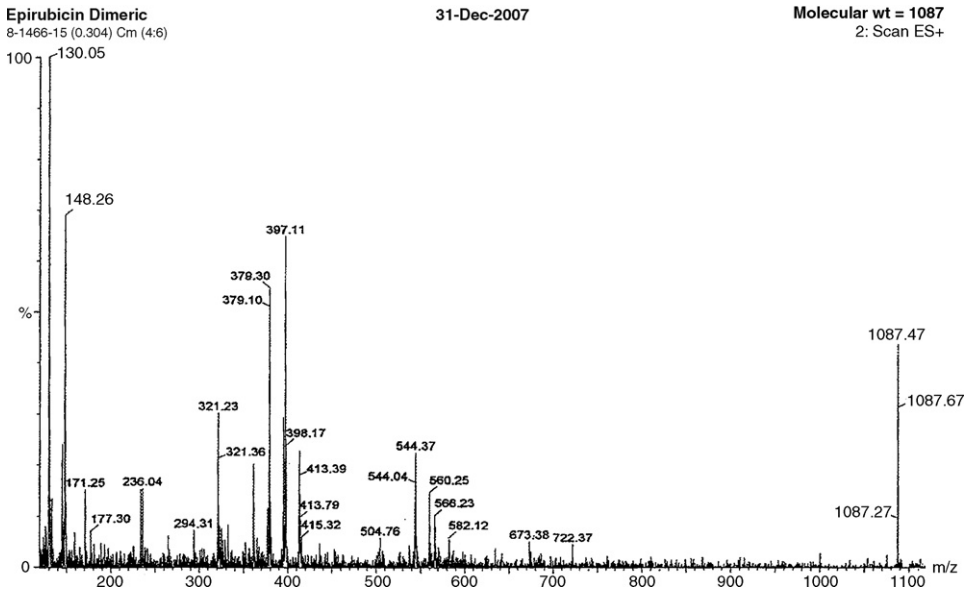


Fig. 2. Epirubicin impurity-1, mass spectrum.

ion $[M+H]^+$ at m/z 1087 indicating that the impurity-1 has molecular mass more than that of epirubicin (543 Da). The fragmentation patterns of protonated molecular ion 1087 (Fig. 3) were obtained as: m/z at 794, 775 and 544 amu due to loss of two molecules of sugar, one molecule of H_2O and one molecule of epirubicin, respectively. The molecular ion and daughter ions indicate that the impurity-1 is dimeric impurity of epirubicin. For further confirmation, the ^1H NMR spectrum of epirubicin impurity-1 (Fig. 4) was found to be same as epirubicin ^1H NMR spectrum, excluding side chain C14 methylene protons signals. In the ^1H NMR spectrum of impurity-1, the C14 methylene protons signals are observed at 3.60 ppm, while in epirubicin hydrochloride, the C14 methylene protons signals are observed at 4.56 ppm. Hence all the spectral data support the epirubicin impurity-1 was found to be as epirubicin dimer that is reported in European pharmacopoeia (supplement 5.6, p. 4574).

3.2. Structure elucidation of epirubicin impurity-2

The ESI mass spectrum of impurity-2 (Fig. 5) gave protonated molecular ion $[M+H]^+$ at m/z 1105. The fragmentation patterns of

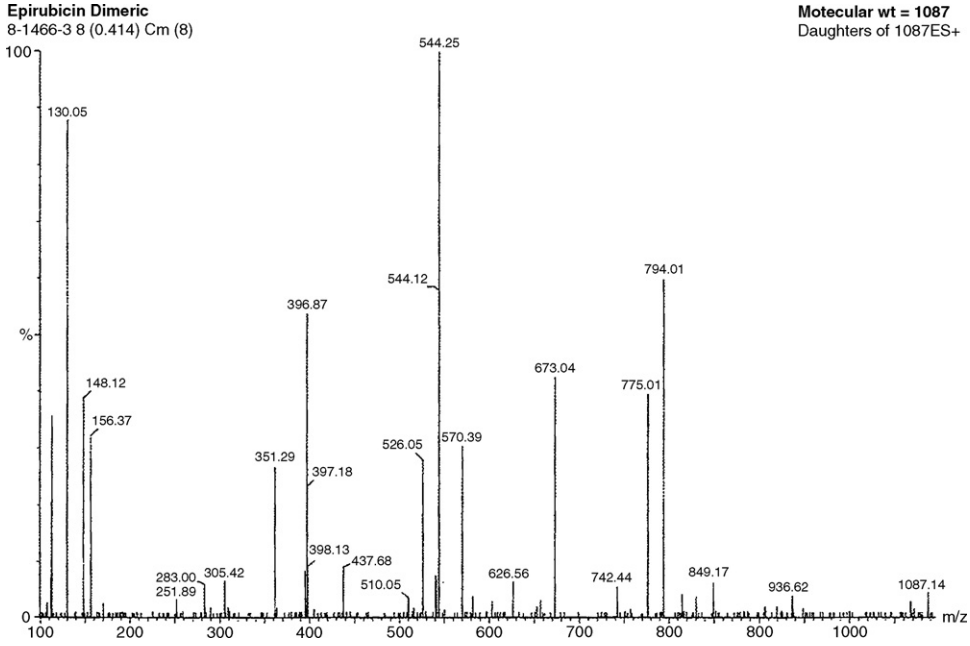


Fig. 3. Epirubicin impurity-1, daughter ion spectrum.

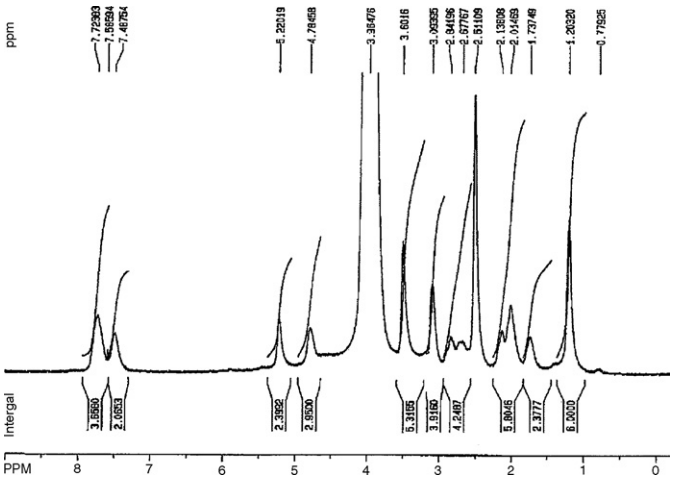


Fig. 4. Epirubicin impurity-1, ^{1}H NMR spectrum.

molecular ion 1105 (Fig. 6) were obtained as: *m*/*z* at 1000, 886, 706, 544, 413 and 397 amu. NMR experiments could not be performed due to lack of sample quantity and stability problem. Structure of this impurity could not be confirmed. We suspect it to be a complex of two epirubicin molecules.

3.3. Structure elucidation of epirubicin impurity-3

The ESI mass spectrum of impurity-3 (Fig. 7) exhibited protonated molecular ion $[M+H]^+$ at *m*/*z* 560. The fragmentation pathway of molecular ion 560 (Fig. 8) was obtained as: *m*/*z* at 542,413, and 337 amu. These *m*/*z* values were obtained due to the loss of one molecule of H_2O, one molecule of sugar and one molecule of sugar plus side chain, respectively. The isolated pure fraction of impurity-3 was heated at 80 °C for 30 min in an acidic aqueous solution. The treated fraction was analyzed by HPLC as per analytical method (Section 2.2). The HPLC showed degradation of impurity-3 into impurity-4. The same degraded fraction was analyzed by LC–MS; the impurity-3 and impurity-4 gave protonated molecular ion $[M+H]^+$ at *m*/*z* 560 and 530, respectively. The ^{1}H NMR spectrum (Fig. 9) showed a close similarity with epirubicin aromatic region, the only difference was found in the side chain, particularly at the C14 methylene proton signals that appear at 6.32 ppm, while in epirubicin hydrochloride, the C14 methylene protons signals are observed at 4.56 ppm. It was observed from LC–MS and NMR data, that no change was found in the ring A, B, C, D and sugar moiety, the only change was found in side chain. All data indicate that the impurity-3 is alpha hydroxy methyl ester of epirubicin, which was converted into impurity-4 (acid molecule) due to hydrolysis of ester side chain. Hence all the Mass, NMR and HPLC data confirms that the epirubicin impurity-3 appears to be a 4-(4-amino-5-hydroxy-6-methyl-tetrahydro-pyran-2-yloxy)-2,5,12-trihydroxy-7-methoxy-6,11-dioxo-1,2,3,4,6,11-hexahydro-naphthacene-2-carboxylic acid hydroxymethyl ester and it was also found to be unknown and previously not reported.

3.4. Structure elucidation of epirubicin impurity-4

The ESI mass spectrum of impurity-4 (Fig. 10) gave sodiated adduct ion $[M+Na]^+$ at *m*/*z* 552 and protonated molecular ion $[M+H]^+$ at *m*/*z* 530. The fragmentation pathway of protonated molecular ion *m*/*z* 530 (Fig. 11) was obtained as: *m*/*z* at 383 due to loss of one molecule of sugar, *m*/*z* 365 due to loss of one molecule of H_2O and *m*/*z* 321 due to loss of one molecule of CO_2, respectively. For further confirmation, the ^{1}H NMR spectrum of epirubicin impurity-4 (Fig. 12) was found to be same as epirubicin ^{1}H NMR spectrum excluding the C14 methylene protons. In the ^{1}H NMR spectrum of Impurity-4, in that the C14 methylene protons were found to be absent, while in epirubicin hydrochloride, the C14 methylene protons were observed at 4.56 ppm. All mass and NMR spectral data indicate that epirubicin impurity-4 is 4-(4-amino-5-hydroxy-6-methyl-tetrahydro-pyran-2-yloxy)-2,5,12-trihydroxy-7-methoxy-6,11-dioxo-1,2,3,4,6,11-

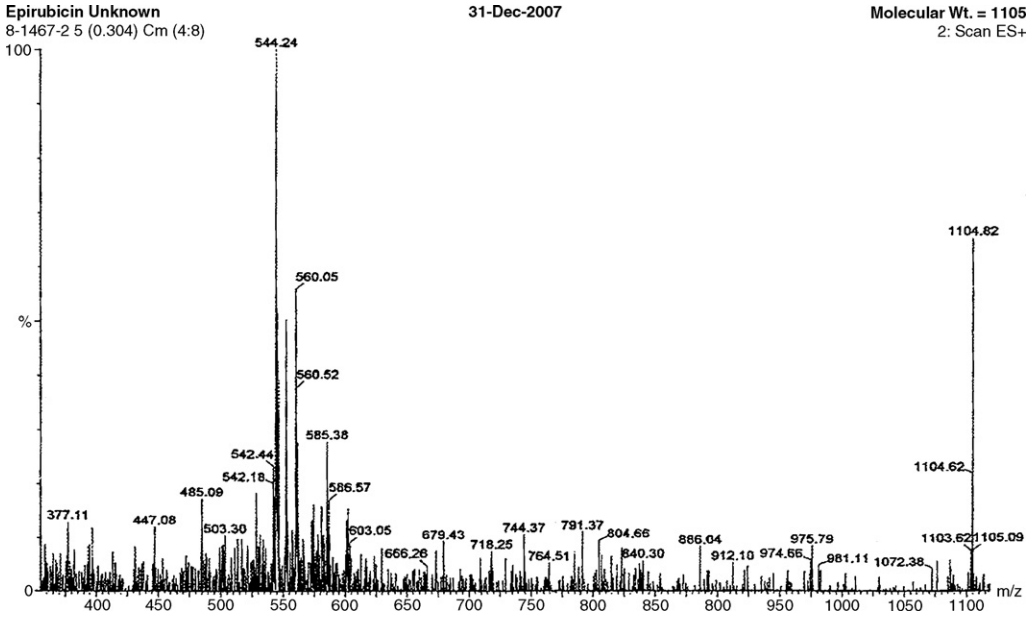


Fig. 5. Epirubicin impurity-2, mass spectrum.

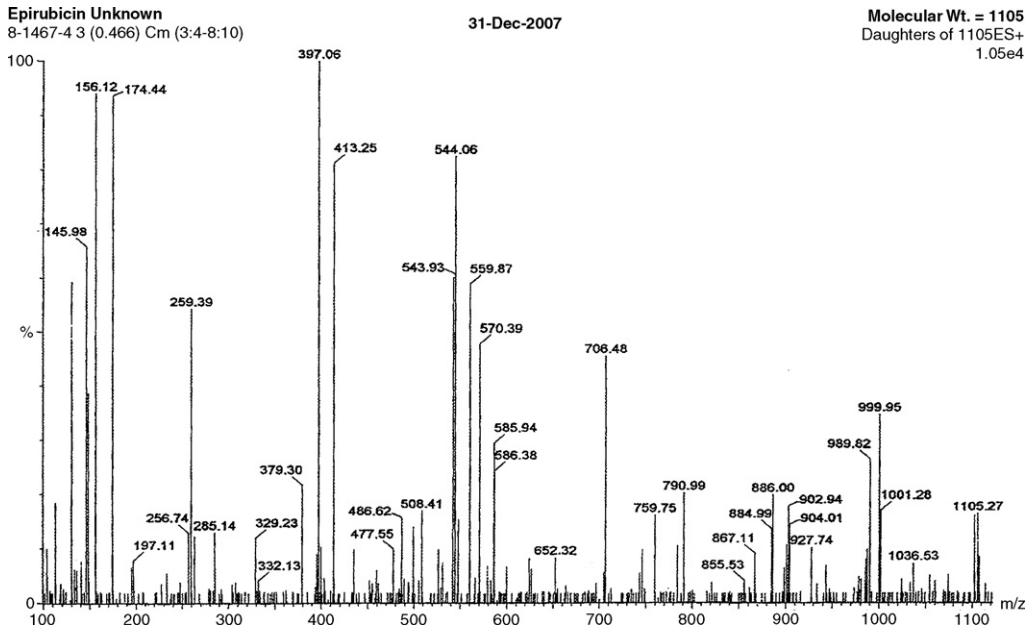


Fig. 6. Epirubicin impurity-2, daughter ion spectrum.

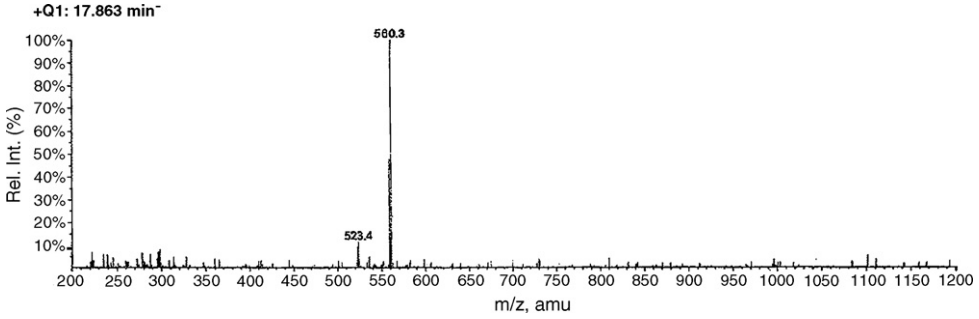


Fig. 7. Epirubicin impurity-3, mass spectrum.

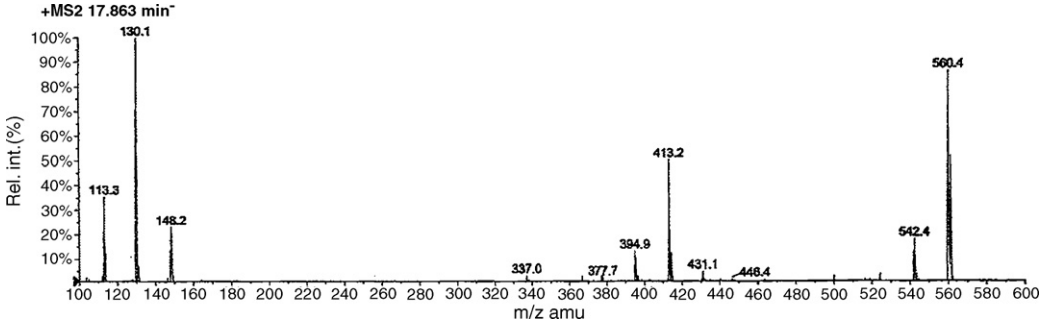


Fig. 8. Epirubicin impurity-3, daughter ion spectrum.

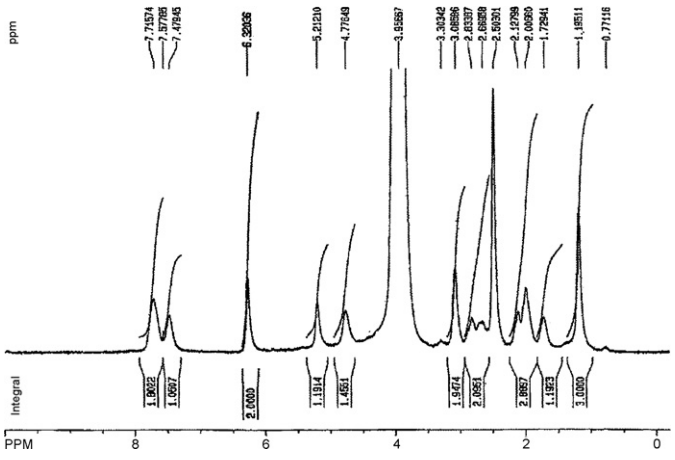


Fig. 9. Epirubicin impurity-3 ^{1}H NMR spectrum.

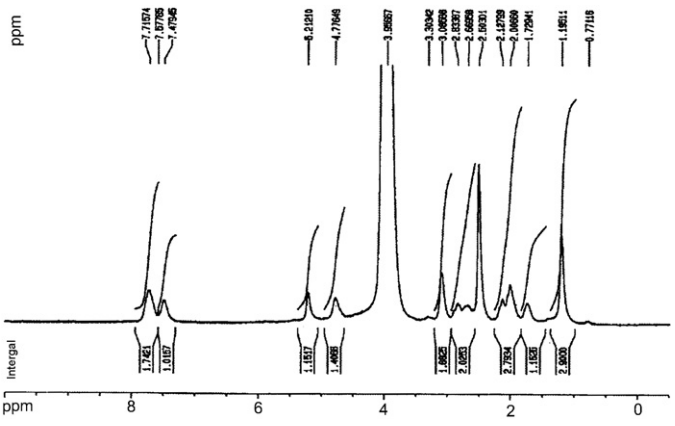


Fig. 12. Epirubicin impurity-4 ^{1}H NMR spectra.

hexahydro-naphthacene-2-carboxylic acid and it was also found to be unknown and previously not reported.

3.5. Postulated mechanism of degradation

The postulated degradation mechanism and impurity formation is shown in Fig. 13. Impurity-1 is formed due to condensation of two molecules of epirubicin. Impurity-3 is formed due to oxidative rearrangement of epirubicin in the side chain in presence of aqueous acidic condition and further this impurity is degraded into impurity-4 due hydrolysis of ester linkage. The impurity-4 is formed due oxidation in the side chain and it is also formed from impurity-3 due to hydrolysis.

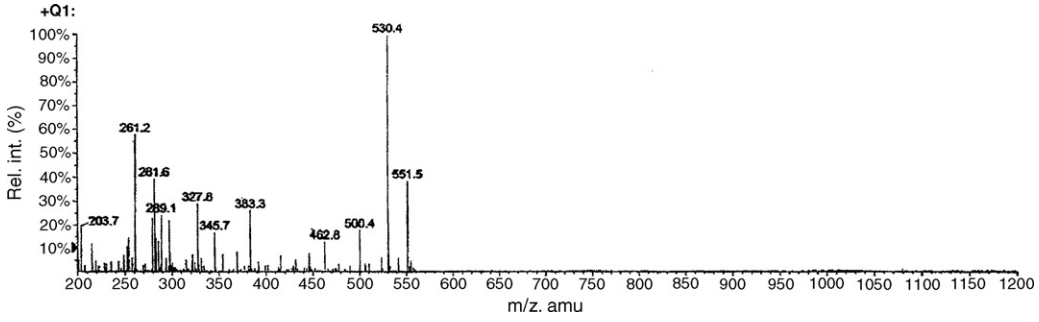


Fig. 10. Epirubicin impurity-4 mass spectrum.

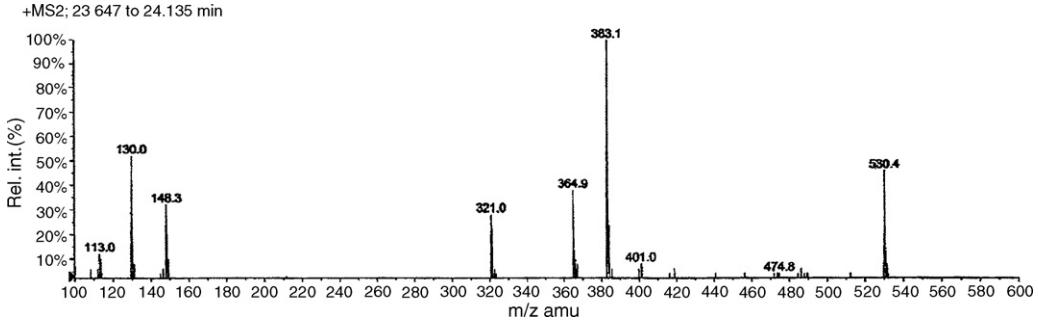


Fig. 11. Epirubicin impurity-4 daughter ion mass spectrum.

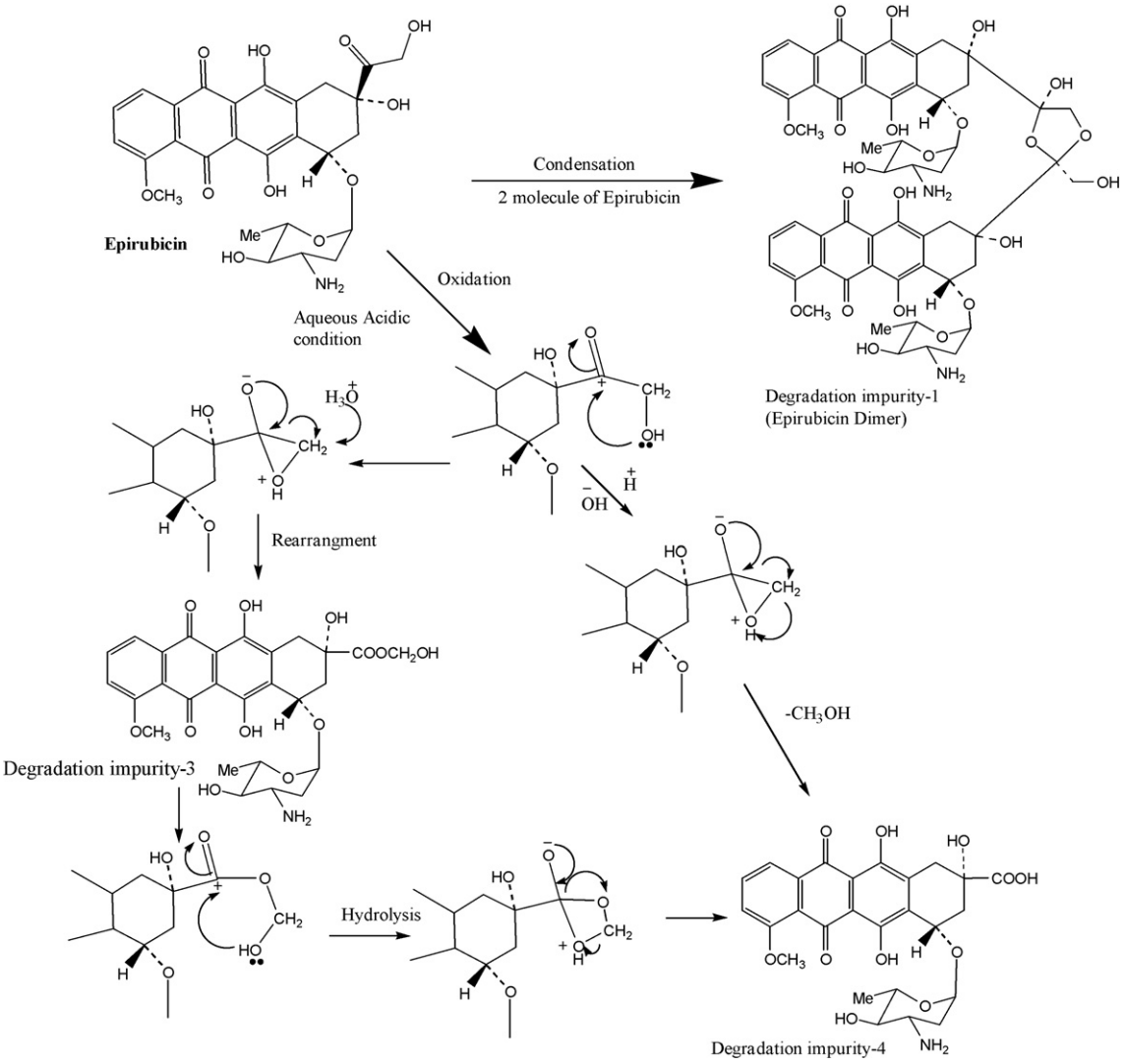


Fig. 13. Postulated mechanism for the formation of epirubicin degradation impurities.

4. Conclusion

Three degradation impurities were isolated and purified using preparative HPLC and subsequently characterized by spectroscopic techniques namely NMR and mass spectrometry. Out of these three degradation impurities, two degradation impurities were found to be novel and characterized as 4-(4-amino-5-hydroxy-6-methyl-tetrahydro-pyran-2-yloxy)-2,5, 12-trihydroxy-7-methoxy-6,11-dioxo-1,2,3,4,6,11-hexahydro-naphthacene-2-carboxylic acid hydroxymethyl ester (impurity-3) and 4-(4-amino-5-hydroxy-6-methyl-tetrahydro-pyran-2-yloxy)-2, 5,12-trihydroxy-7-methoxy-6,11-dioxo-1,2,3,4,6,11-hexahydro-naphthacene-2-carboxylic acid (impurity-4).

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