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RESEARCH ARTICLE

Identification, Isolation and Characterization of Unknown Impurity in Daunorubicin Hydrochloride

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Abstract: An unknown impurity in the bulk drug daunorubicin hydrochloride observed at 1.79 RRT was detected by the means of reverse phase isocratic High performance liquid chromatography (HPLC). This impurity was isolated, enriched and was subjected to mass and NMR spectral studies. Based on the spectral data the impurity was identified as (8*S*,10*S*)-8-ethyl-10-[(2*S*,4*S*,5*S*,6*S*)-4-amino-5-hydroxy-6-methyl-oxan-2-yl]oxy-6,8,11-trihydroxy-1-methoxy-9,10-dihydro-7*H*-tetracene-5,12-dione also known as 13-deoxy-daunorubicin.

Keywords: Daunorubicin, Unknown impurity, Chromatography, Preparative HPLC, Lyophilization, NMR, LC-MS, LC/MS/MS.

Introduction

Daunorubicin[1-5] chemically known as (8*S*,10*S*)-8-acetyl-10-[(2*S*,4*S*,5*S*,6*S*)-4-amino-5-hydroxy-6-methyl-oxan-2-yl]oxy-6,8,11-trihydroxy-1-methoxy-9,10-dihydro-7*H*-tetracene-5,12-dione as its hydrochloride is an anti-cancer ("antineoplastic" or "cytotoxic") chemotherapy drug. It is most commonly used to treat specific types of leukaemia mainly acute myeloid leukemia and acute lymphocytic leukemia[6-10]. It was initially isolated from Streptomyces peucetius. Daunorubicin hydrochloride is commonly obtained fermentatively from a modified strain of Streptomyces peucetius. Official monographs appearing in both the USP[11] and European pharmacopoeia[12] are available for the quality control of daunorubicin hydrochloride.

Impurity profile of a drug substance is critical to its safety assessment and manufacturing process. For safety reasons, the impurities that exceed 0.1% in a drug must be identified prior to clinical trials (International Conference on Harmonization, 2006[13]). The present study provides the isolation and characterization of an unknown impurity appearing above the ICH limit in the final API samples. The said impurity was isolated using preparative HPLC and characterized by using NMR and MS spectral data.

Experimental

Daunorubicin hydrochloride prepared in house was used for the experiments while sodium lauryl sulfate (AR grade), orthophosphoric acid 85% (AR grade), trifluroacetic acid (AR grade) and acetonitrile (HPLC grade) were procured from Merck. Highly pure Milli Q Water obtained from Millipore Milli-Q plus purification system was used wherever aqueous preparations were involved.

Analytical conditions

The chromatographic separation was performed on Waters Alliance 2695 separation module with 2487 dual λ absorbance detector. The data were processed using Empower 2.0 software. The analytical conditions used were, Inertsil ODS-2, 250 X 4.6 mm, 5 µm column, with a flow rate 1.0 mL/min, mobile phase was a mixture of equal volumes of acetonitrile and a solution containing 2.88 g/L of sodium lauryl sulfate and 2.25 g/L of phosphoric acid and the detection was performed at 254 nm. Final concentration of sample solution prepared in mobile phase for HPLC injection was 1000 ppm. The HPLC chromatograms were integrated for the detected impurity and main peaks while peaks due to blank were not integrated.

Isolation of Impurity

The observed unknown impurity was isolated from daunorubicin hydrochloride sample by using preparative HPLC. The preparative LC system used was Waters Delta prep preparative chromatography system. For the isolation Waters X-Bridge prep C^{18}, 5 µm OBD, 30 X 50 mm column was used. The isocratic Mobile phase was used which was a 65:35 mixture of buffer (0.1% Trifluoroacetic acid solution) and acetonitrile. The flow rate was set at 10.0 mL/min and the effluent was monitored at 254 nm. Concentration of 10 mg/mL was used. Total 30 runs were performed using 10 mL (10 mg) in each loading on to the preparative LC column. The major peaks were isolated individually. All isolated fractions having maximum purity were combined and reanalyzed on analytical HPLC. The combined fractions were concentrated under high vacuum using Buchi rotavapor R-124. The concentrated fractions were then lyophilized using freeze dryer (Instrument model Vertis) and the lyophilized sample was used for identification purpose after a final integrity check on analytical HPLC.

LC-MS/MS analysis

LC-MS was carried out on Waters LC-DAD-q-TOF premier equipped with Electrospray ionization source. Instrument control and data were performed with the help of Mass Lynx 4.1 software. The samples were infused into the ion source chamber with a T- junction delivering approximately 1/3 of the flow to the mass spectrometer. The ion source temperature was 300 ^{O}C and the ESI needle voltage was set at 3.0 kV. Nitrogen gas was used as the drying gas which was maintained at a flow rate of 10 mL / min. The collision energy was set between 5.0 and 10.0 to maximize the ion current in the spectra. The spray voltage was 3.0 kV. The temperature of the capillary was 300 ^{o}C. Ions were generated in the collision cell wherein Argon was used as the collision gas. For this experiment mass compatible gradient HPLC Method (Table 1) was developed and used for the separation of unknown impurity. Test preparation was 1000 ppm with methanol as a diluent. Injection volume was set to 50 µL.

NMR Spectroscopy

NMR experiments were performed on Bruker Avance II plus 500 MHz FT-NMR spectrometer with deuterated solvent DMSO-d_6 as a diluent at ambient temperature. The chemical shift values were reported on the δ scale in ppm. The broad peak appearing at δ 3.3 due to traces

Deuterated water (HOD) and δ 2.5 due to traces undeuterated DMSO were taken as the internal standards for assigning the δ values in the recorded ^{1}H NMR Spectra's while septet appearing at δ 39.51 for DMSO was taken as the internal standard for assigning the δ values in the recorded ^{13}C NMR spectra's

Table 1. Gradient HPLC method

Time minutes	Mobile phase A (0.2% Triflouroacetic acid)	Mobile phase B (Acetonitrile)	Flow
0.00	75	25	1.0
5.00	75	25	1.0
10.00	70	30	1.0
25.00	70	30	1.0
30.00	50	50	1.0
40.00	50	50	1.0
45.00	75	25	1.0
50.00	75	25	1.0

Results and Discussion

The present investigation describes the isolation and characterization of the unknown impurity as observed at ~1.79 RRT during the HPLC analysis of manufactured daunorubicin hydrochloride batches. The representative HPLC chromatogram of daunorubicin hydrochloride exhibiting the unknown impurity at RRT 1.79 is shown in Figure 1.

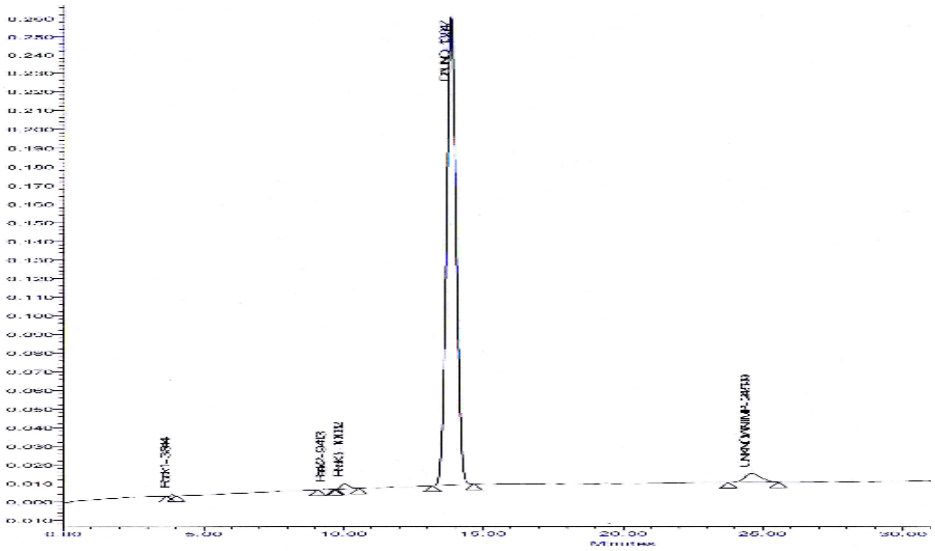

Figure 1. Representative HPLC chromatogram of daunorubicin HCl

LC-MS/MS of daunorubicin sample enriched in unknown impurity

LC-MS followed by MS/MS of the observed unknown impurity showed major molecular ion at 514 indicating a molecular weight of 513 for the impurity. Further MS/MS of the main molecular ion showed two daughter ions at m/z 367 and 349. The MS/MS pattern as observed for the impurity showed similarity with the MS/MS pattern of daunorubicin hydrochloride indicating that the basic structure of the impurity was similar to daunorubicin hydrochloride. A detailed study of the fragmentation patterns of the parent drug served as a template to elucidate the structure of unknown impurity by comparison of their fragmentation

pathways and neutral losses. Mass analysis established that the molecular weight of the impurity was less by 14 amu than daunorubicin [Mol. wt. 527]. The LC as obtained for the impurity enriched Daunorubicin is depicted in Figure 2A which showed major daunorubicin peak at ~20 minutes and the unknown impurity at 31.76 minute and MS spectra of the observed two peaks are depicted in Figure 2B and 2C

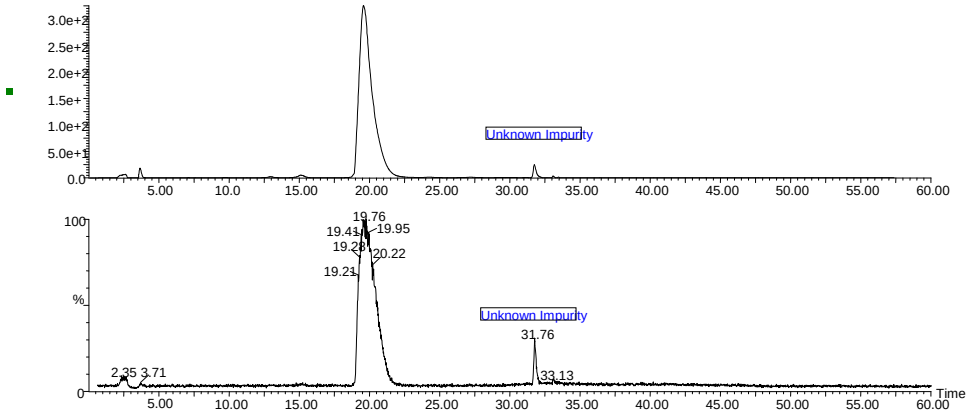

Figure 2A. LC as obtained during the LC-MS experiment of impurity enriched daunorubicin sample

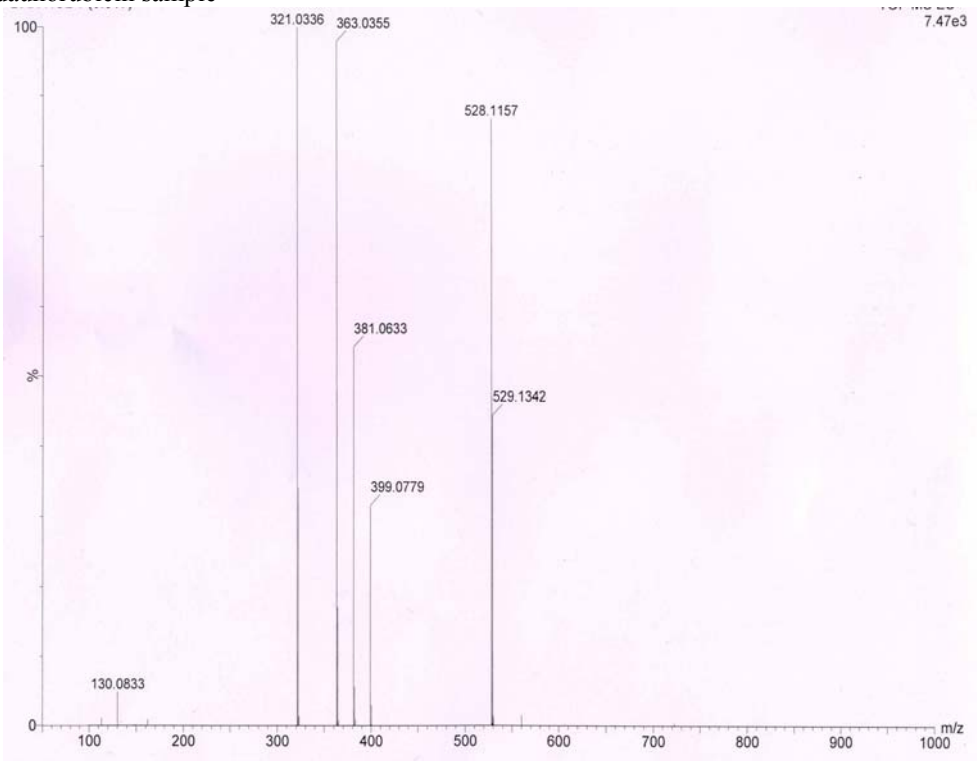

Figure 2B. MS Spectra of daunorubicin eluting at 20.0 minutes in LC

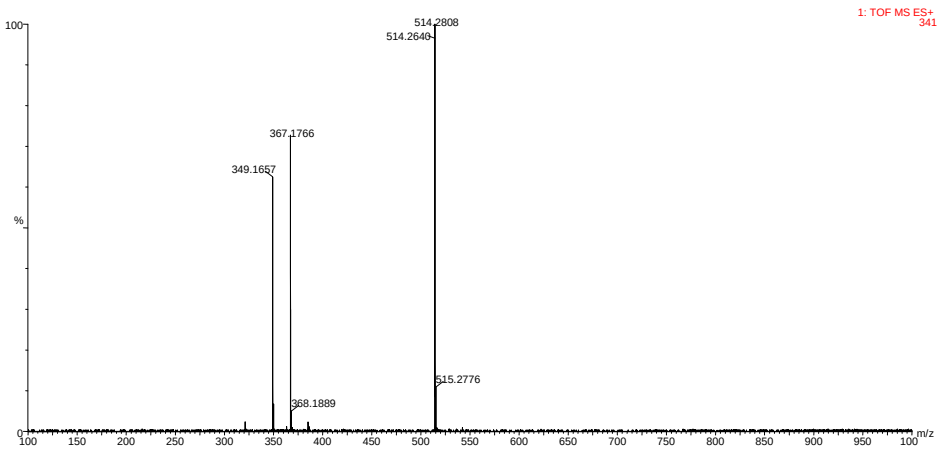

Figure 2C. MS Spectra of unknown impurity eluting at 31.76 minutes in LC

Isolation of impurity

Unknown impurity was isolated by prep HPLC. All impurity fraction collected from prep HPLC was concentrated and after lypophilization about 20 mg of unknown impurity was obtained. This impurity was injected in HPLC method for checking its integrity and was found to be more than 95% pure. For details refer HPLC chromatogram of the isolated Impurity as depicted in Figure 3.

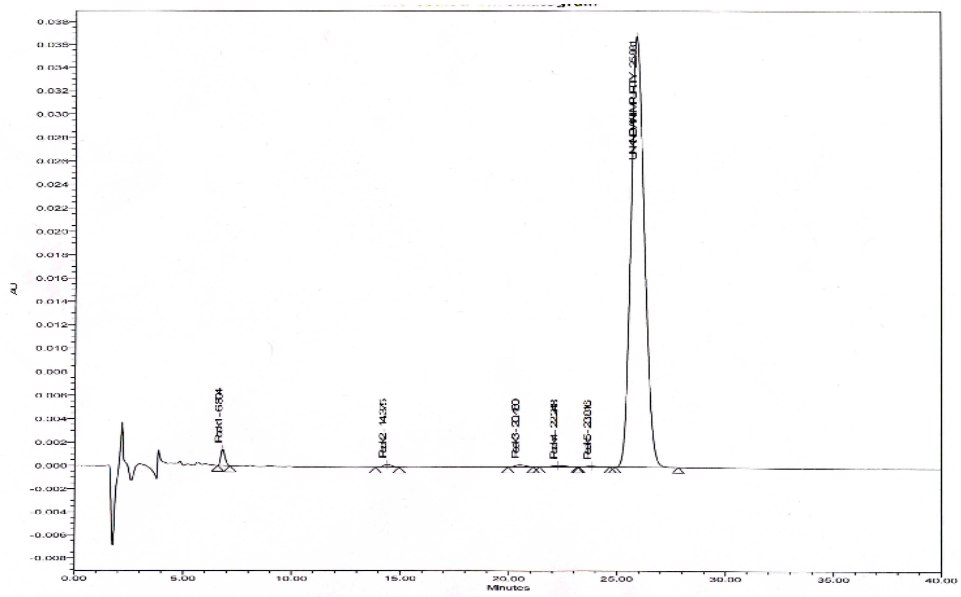

Figure 3. HPLC chromatogram of isolated unknown impurity

Structure elucidation of unknown impurity

The High Resolution Mass Spectral data of the unknown impurity showed similar pattern as obtained during LC-MS experiments that is it showed a major molecular $[M+H]^+$ ion at m/z 514 [(calculated 513

for $C_{27}H_{31}NO_9$) which corresponded to the molecular formula $C_{27}H_{31}NO_9$. Besides it showed two daughter ions at m/z 367 and 349. A molecular mass of 513 indicated that the structure was closely associated to duanorubicin.

^{1}H NMR data for the impurity (Figure 4b,) was compared with those of daunorubicin hydrochloride (Figure 4a). In the ^{1}H NMR of the impurity signal corresponding to 3 protons of methyl group [CH_3CO] at δ 2.280 was absent while two new signals one at δ 0.0936 corresponding to 3 protons of a diamagnetic group probably aliphatic methyl group [-CH_3] and the other at a slightly up field region at δ 1.728 probably corresponding to 2 protons of an paramagnetic aliphatic methylene group [=CH_2] were observed. This indicated that probably the impurity structure was having a different group in the 8 position which was acetyl in the case of daunomycin.

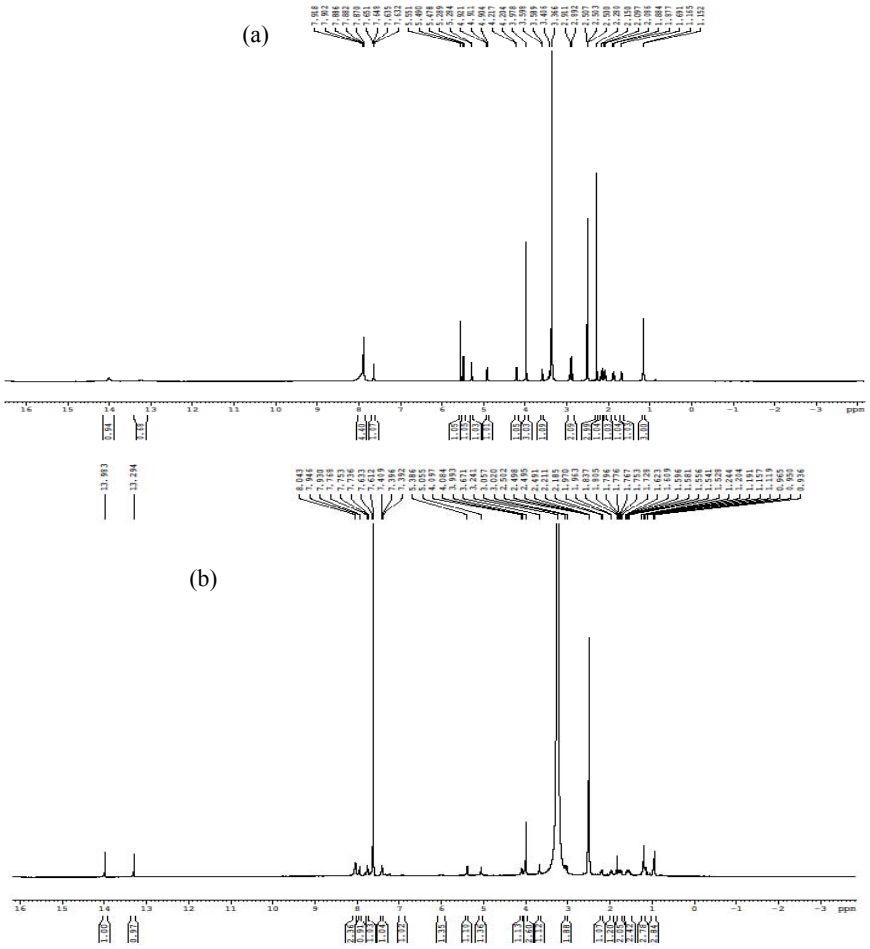

Figure 4. ^{1}H NMR Spectra of (a) Daunomycin (b) Impurity

Thus further ^{13}C NMR data for the impurity (Figure 5a & b and Figure 6a & b) was compared with those of daunorubicin hydrochloride. It was observed that in the ^{13}C NMR of unknown impurity the C=O signal was not observed at δ 212.39 proving its absence. The presence of a new up field methyl carbon [$\underline{C}H_3$] signal at δ 7.77 and a new signal at δ 38.76

was observed. The signal at δ 7.77 was confirmed as aliphatic methyl carbon while the signal at δ 38.76 was confirmed as an aliphatic methylene carbon by DEPT-135 experiment. This confirmed that the impurity was having a ethyl group [$–CH_2$-CH_3] in the 8 position instead of the Acetyl group as observed in the daunorubicin strucure. Further ^{13}C & DEPT-135 experiment also confirmed the rest of the carbon skeleton similar to daunomycin. Details of ^{1}H & ^{13}C NMR are tabulated in Table 2 & 3.Also the absence of oxygen in the impurity structure was justified by the molecular mass as obtained for the impurity.

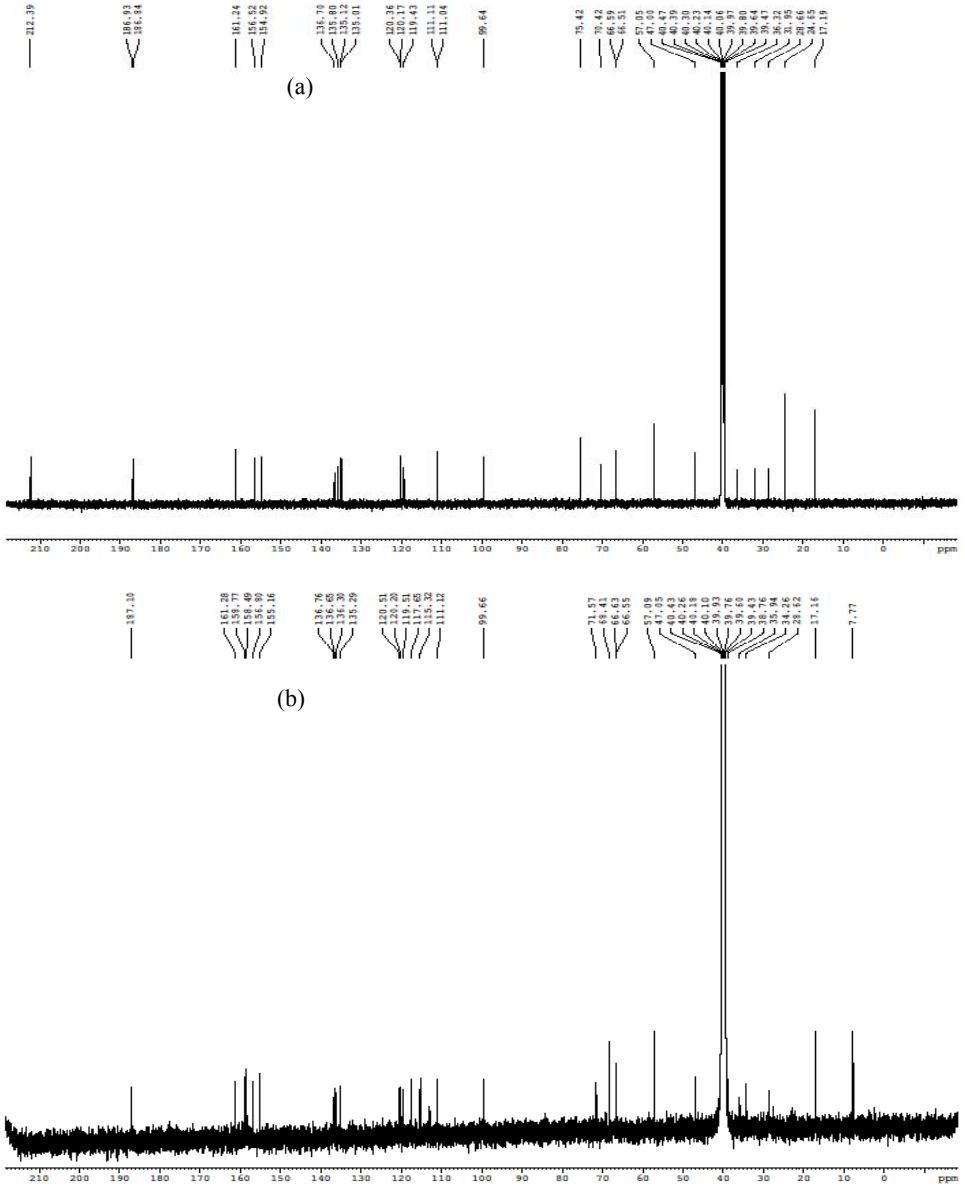

Figure 5. ^{13}C NMR Spectra of (a) Daunomycin (b) Impurity

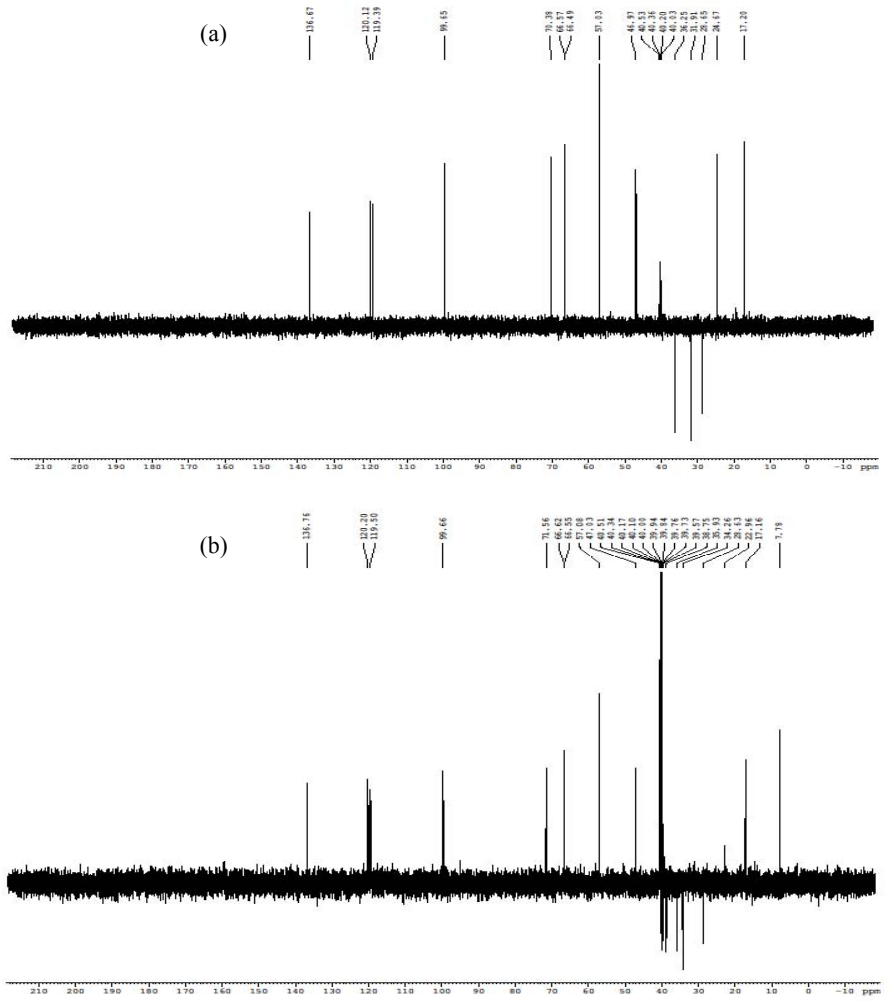

Figure 6. DEPT 135 NMR Spectra (a) Daunomycin (b) Impurity

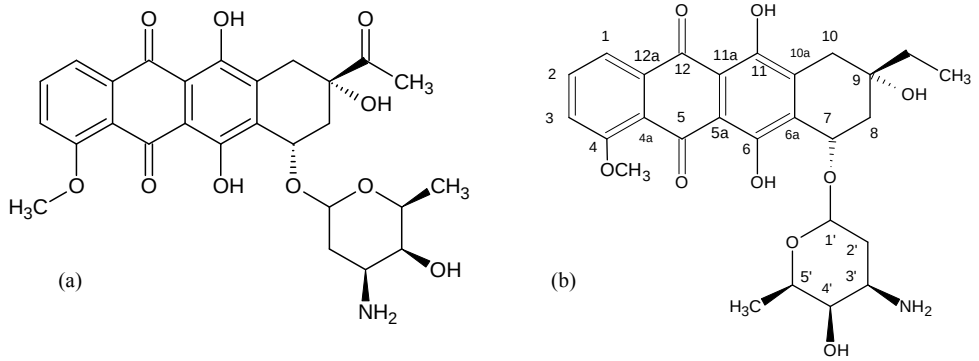

Figure 7. Chemical Structure of a) Daunomycin; b) Impurity

Table 2. ^{1}H NMR (500 MHz, DMSO – d_6) assignments for impurity

S. No	Protons	Shift, ppm	Multiplicity	No. of H
1	1-H	7.736	Doublet	1
2	2-H	7.946	Doublet	1
3	3-H	7.39	Multiplet	1
4	7-H	5.00	Multiplet	2
5	8-H	1.968	Multiplet	2
6	10-H	3.02	Multiplet	2
7	1'-H	5.38	singlet	1
8	2'-H	1.528	Multiplet	2
9	3'-H	3.50	Multiplet	1
10	4'-H	3.671	Multiplet	1
11	5'-H	4.084	Multiplet	1
12	5'-CH_3	1.15	Doublet	1
13	4-OCH_3	3.978	Singlet	3
14	9-$C\underline{H}_2CH_3$	1.728	Multiplet	2
15	9-$CH_2C\underline{H}_3$	0.093	Triplet	3
16	9-O<u>H</u>	8.043	Singlet	1
17	11-O<u>H</u>	13.294	Singlet	1
18	6-O<u>H</u>	13.983	Singlet	1

Table 3. ^{13}C NMR Assignments for Impurity

S.No	Carbon	Shift, ppm
1	C-1	119.51
2	C-2	136.76
3	C-3	117.65
4	C-4	161.28
5	C-5	187.10
6	C-6	156.80
7	C-7	66.63
8	C-8	35.94
9	C-9	71.57
10	C-10	34.26
11	C-11	136.65
12	C-12	158.77
13	C-12a	136.30
14	C-4a	115.32
15	C-5a	111.12
16	C-11a	110.12
17	C-6a	120.51
18	C-10a	135.29
19	9-$\underline{C}H_2CH_3$	38.76
20	9-$CH_2\underline{C}H_3$	7.77
21	4-OCH_3	57.09
22	C-1'	99.66
23	C-2'	28.62
24	C-3'	47.05
25	C-4'	68.41
26	C-5'	66.55
27	C-5'-CH_3	17.16

Thus from all the mass and NMR spectral data the impurity was found to be (8*S*,10*S*)-8-ethyl-10-[(2*S*,4*S*,5*S*,6*S*)-4-amino-5-hydroxy-6-methyl-oxan-2-yl]oxy-6,8,11-trihydroxy-1-methoxy-9,10-dihydro-7*H*-tetracene-5,12-dione or 13-deoxy-daunorubicin (Figure 7). The structure so identified also justified the mass fragmentation pattern as represented in Figure 8.

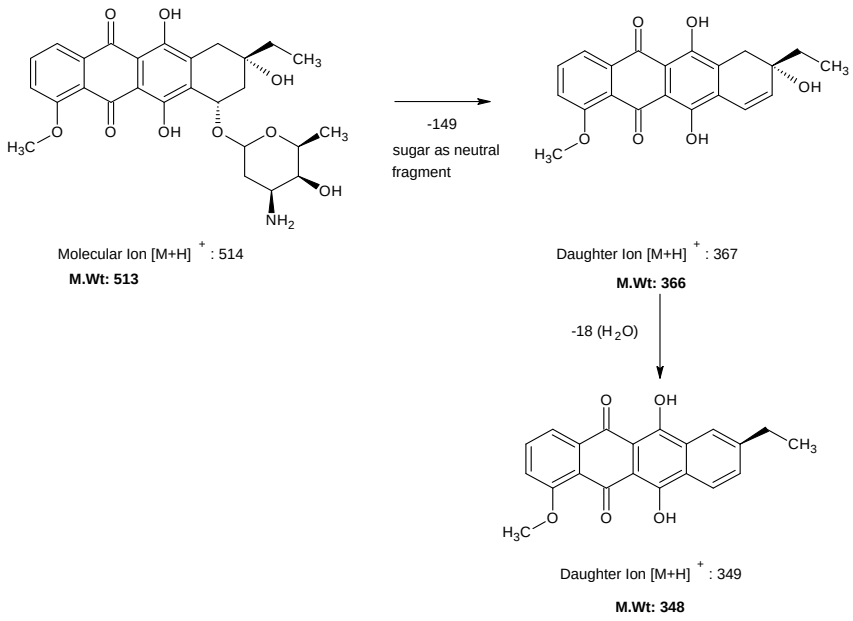

Figure 8. Mass fragmentation pattern of Impurity

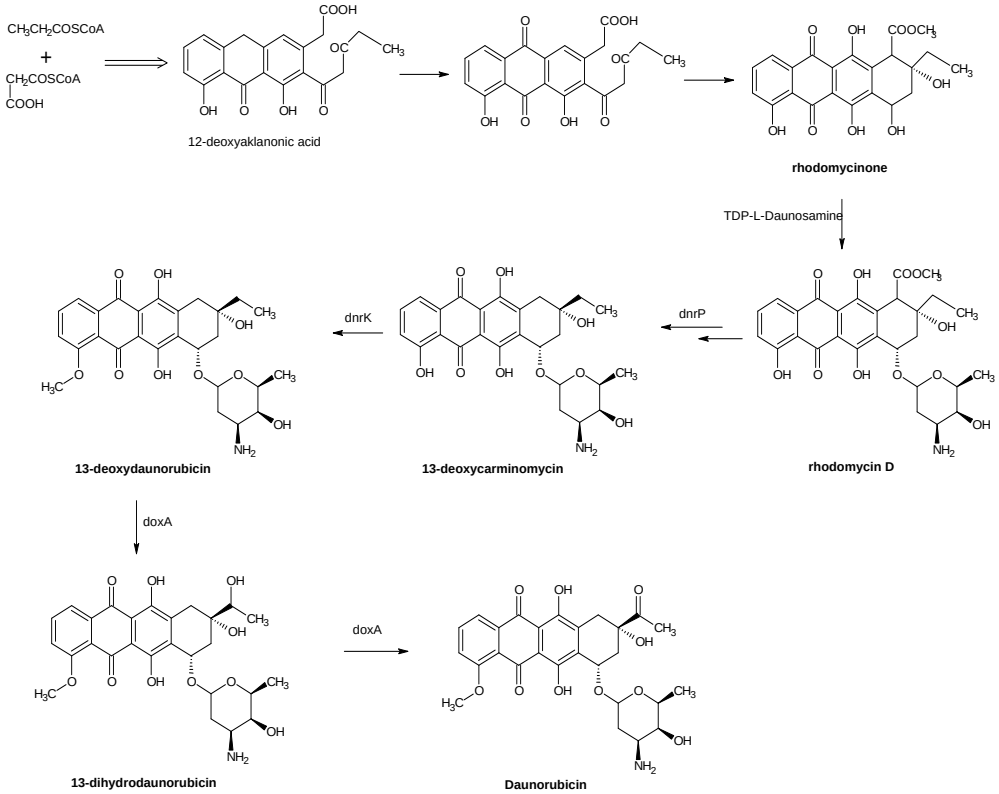

Figure 9. Abbreviated pathway for bio-synthesis of daunorubicin

Probable source of impurity

Daunorubicin hydrochloride is commonly synthesized fermentatively. An outline of the fermentation biosynthetic pathway[14] leading to daunorubicin is depicted in Figure 9. By analyzing both the bio-synthetic pathway as depicted as well as the confirmed impurity structure as identified it can be concluded that the impurity (13-Deoxy-daunorubicin) is an integral part of the fermentative biosynthetic pathway appearing as one of the intermediates which is further converted to daunorubicin. Thus it is this intermediate which is a precursor to daunorubicin which appears as an impurity due to its incomplete bio conversion to daunorubicin.

Conclusion

The unknown impurity observed at ~1.79 RRT during HPLC analysis of some of the in-house manufactured Daunorubicin hydrochloride batches was isolated using preparative HPLC and subsequently characterized by spectroscopic techniques namely NMR and mass spectrometry. The impurity was found to be (8*S*,10*S*)-8-ethyl-10-[(2*S*,4*S*,5*S*,6*S*)-4-amino-5-hydroxy-6-methyl-oxan-2-yl]oxy-6,8,11-trihydroxy-1-methoxy-9,10-dihydro-7*H*-tetracene-5,12-dione or 13-deoxy-daunorubicin which probably is the bio-synthetically unconverted intermediate.

Acknowledgement

We are thankful to our analytical section which contributed to the maximum in isolating and characterizing the impurity.

References

1. Isolation from fermentation broths of Streptomyces puecetius: Cassinelli G, Orezzi P, Microbio G, 1963, **11**, 167, C. A.1965, **62**, 9482b.
2. Di Marco A, Gaetani M, Orezzi P, Scarpinato B M, Silvestrini R, Soldati M, Dasdia T and Valentini L, *Nature,* 1964, **201**, 706-707; DOI:10.1038/201706a0
3. Sylvie Pinnert, Leon Ninet and Jean Preud'Homme, US 3997662 A.
4. Arcamone F, Franceschi G, Orezzi P, Cassinelli G, Wanda Barbieri and Rosanna Mondelli, *J Am Chem Soc.,* 1964, **86(23)**, 5334-5335; DOI:10.1021/ja01077a059
5. Iwamoto R H, Lim P and Bhacca N S, *Tetrahedron Lett*., 1968, **36**, 3891.
6. Edward M Acton, Allan N Fujiwara and David W Henry, *J Med Chem*., 1974**, 17**, 659.
7. Tan C, Tasaka H, Yu KP, Murphy ML and Karnofsky D A, *Cancer,* 1967, **20(3),** 333–353.
8. White R J and Stroshane R M, *Drug Pharm. Sci*., 1984, **22**, 569-594.
9. Momparler R L, Karon M, Siegel S E and Avila F, *Cancer Res*., 1976, **36(8),** 2891–5.
10. Riggs C E Jr Metabolism and clinical pharmacokinetics, *Semin Oncol*., 1984, **11**, Suppl. 3, 2-11.
11. United State Pharmacopoeia 36, 3147-3148.
12. European Pharmacopoeia, 7th Edn., 1792-1793.
13. ICH Harmonised Tripartite Guideline Impurities in New Drug Substances Q3A(R2) Current Step 4 version dated 25 October 2006.
14. Natalie N, Sharee L O *et al., J Bacteriol*., 1999, **181(1)**, 305-318.