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Characterization of Doxorubicin Unknown Impurity using 1D-NMR (1H) Spectroscopy and LC-MS Mass Ionization

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Abstract

Doxorubicin, an anthracyline medication, is regularly used to treat a number of malignancies, including sarcoma, lung, breast, gastric, thyroid, non-Hodgkin's lymphoma, ovarian, Hodgkin's lymphoma, multiple myeloma, and childhood cancers. One unidentified doxorubicin impurity with an RRT of 1.23 and a concentration higher than 0.1% was discovered during the quality inspection of doxorubicin in bulk forms. By thoroughly analysing the 1D-NMR (1H) spectroscopic data and the LC-MS mass ionization spectrum data, the structure and molecular mass of the unknown doxorubicin impurity with an RRT of 1.23 were determined. From spectroscopic data and the LC-MS mass ionization spectrum data, it was also observed that doxorubicin and unknown doxorubicin impurity with an RRT of 1.23 are interconvertible.

Keywords: Doxorubicin; RRT impurity 1.26; 1D-NMR (1H); LC-MS mass ionization; Structure elucidation

1. Introduction

An antibiotic titled doxorubicin is obtained from the bacteria Streptomyces peucetius. It is extensively included as a chemotherapy medication [1]. Doxorubicin belongs to the class of chemotherapy medications designated as anthracyclines. Doxorubicin may well be used to treat: soft tissue sarcomas, bone sarcomas, cancers associated to small cell lung, breast, bladder, ovary, and thyroid, leukemias of acute lymphoblastic and acute myeloblastic and Hodgkin lymphoma [2-5]. Doxorubicin's main method of operation is its capacity to intercalate among DNA base pairings, breaking DNA strands thereby inhibiting the creation including both DNA as well as RNA. Topoisomerase -2 is suppressed by doxorubicin, which culminates in DNA destruction and the activation of apoptosis. Doxorubicin also results in free radical facilitated oxidative deterioration to DNA when paired with iron, severely restricting DNA synthesis [6].

Impurities could have an influence on the upkeep of medicine quality, safety, including effectiveness. The impurity controlling strategies, both physical and also chemical, switches through entire inspects of drug medication development practice. Four doxorubicin deterioration (oxidation-based) products were identified by Dheeraj and Gulshan [7]. They characterized those employing LC-MS-(+)ESI mass spectral of doxorubicin and LC–MS–TOF doxorubicin mass spectral. The products characterized by Dheeraj and Gulshan were: "9-desacetyldoxorubicin", "9-desacetyldoxorubicin-9-hydroperoxide", "3-hydroxy-9-desacetyldoxorubicin-9-hydroperoxide', and "1-hydroxy-9-desacetyldoxorubicin-9-hydroperoxide".

In tests on the quality examination of doxorubicin in bulk forms, one unidentified impurity, having RRT of 1.23, with a concentration exceeding 0.1% was found. We named this as RRT Impurity 1.26. In accordance with "ICH Q3 B (R2)", the quantity limit of unidentified impurity more than 0.1% must be identified and characterized [8]. For each finished

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product, an impurity characteristic analysis needs be conducted in order to locate and characterize whatever unknown impurities prevalent at levels even smaller than 0.05%. In light of the strict criteria from the regulatory organizations, it is imperative to identify as well as characterize the impurities in the completed product. So, with our expertise in analysis of impurities[9,10] the present work was taken up.

Prior to being purified through preparative column HPLC, RRT Impurity 1.26 was first separated utilizing column chromatography. Additionally, it was crucial to check the hypothesized structure of RRT Impurity 1.26 as well as to deduce its potential structure using LC-MS and NMR investigations. For the first time, the RRT Impurity 1.26 has been isolated, its structure was characterized in this publication. This study also provides the first account of the procedure for isolating RRT Impurity 1.26.

2. Materials and methods

2.1. Chemicals

Unless otherwise noted, all of the chemicals are indeed analytical-reagent standard. Water employed herein investigation was de-ionized and glass-distilled. Herein investigation, acetonitrile, CD3OD (deuterated methanol), and formic acid were used, all from Merck Limited in India. The doxorubicin investigated herein investigation was from Hetero Drugs, Hyderabad, Telangana.

2.2. Instrumentation

This included: Shimadzu LC-8A separation segment (Japan) connected to a Shimadzu UV detector (Japan) for RRT Impurity 1.26 isolation; Software loaded in above equipment was "LC solutions" software for RRT Impurity 1.26 data interpretation; Luna column C18 (250 mm Length, 50 mm identification, 10 µm sized particle) for RRT Impurity 1.26 separation; Single quadrupole Agilent 6120 LC/MS apparatus for RRT Impurity 1.26 characterization; analyst version software 1.6.2 for RRT Impurity 1.26 LC-MS data interpretation; ESI for RRT Impurity 1.26 ionization source; NMR 400 MHz spectrometer (Bruker, Switzerland) for RRT Impurity 1.26 structure characterization.

2.3. HPLC conditions to isolate RRT Impurity 1.26

The formic acid (0.05%) in water and formic acid (0.05%) in acetonitrile served as the mobile phases I and II, respectively, for the lined gradient manner elution, which was managed to flow in Luna column C18 (250 mm Length, 50 mm identification, 10 µm sized particle) at 60.0 mL/min, 25 °C, with UV detection at 234 nm. The lined gradient manner elution was as follows (Table 1):

Table 1

Lined gradient manner elution for HPLC (preparative)

Time (min)	% of formic acid (0.05%) in water	% of formic acid (0.05%) in acetonitrile
0.00	85	15
5.0	85	15
12.0	75	25
17.0	75	25
23.0	70	30
35.0	10	90

Prior to getting injected, the mobile phases have all been ultrasonically degassed for 15 min after being processed through one Millipore filter membrane. The samples were then administered (6 ml) by an auto-sampler.

2.4. Doxorubicin sample preparation

A 300.0 mg quantity of the doxorubicin sample were precisely weighed, and then 6 ml of diluent (mobile phase) were added. Sonicated to dissolve doxorubicin sample, then thoroughly blended.

2.5. Isolation of RRT Impurity 1.26

Injected 6 ml of doxorubicin sample to Luna column C18 (250 mm Length, 50 mm identification, 10 µm sized particle). The conditions in section "HPLC conditions to isolate RRT Impurity 1.26" were followed.For each injection of doxorubicin sample (6 ml), collected the impurity fractions into several volumetric flasks at about 27 to 29 min elution durations, and stored immediately all fractions promptly at -80°C.

2.6. ^{1}H NMR analysis conditions

An NMR 400 MHz spectrometer (Bruker, Switzerland) was involved in RRT Impurity 1.26 structure characterization. The ^{1}H NMR spectra for RRT Impurity 1.26 were produced by completing 16

scans and documenting them with quite a 1 s pulse repetitive time while using a flipping angle 30°. The deuterated solvent employed in NMR investigations of RRT Impurity 1.26 was CD_3OD (deuterated methanol).

2.7. Sample preparation for 1H NMR investigations of RRT Impurity 1.26

The sample RRT Impurity 1.26 was developed in CD_3OD (deuterated methanol) at a quantity of 5 mg/mL.

2.8. ^{1}H NMR investigation

With regard to CD3OD, the chemical shifts of 1H NMR for RRT Impurity 1.26 were quantified using a delta magnitude in ppm. The peak at 3.3 ppm, which represents the remaining methanol used forRRT Impurity 1.26 dilution, was utilized as a benchmark for the protons' chemical changes. With the sample rotating at a speed of 200 MHz, all spectra for RRT Impurity 1.26 were recorded.

2.9. LC-MS analysis conditions

The analyses of RRT Impurity 1.26 and doxorubicin were performed on a Omega Luna C18 column (50 mm Length, 2.1 mm identification, 1.6 µm sized particle) using a lined gradient manner elution of formic acid (0.1%) in 5% methanol (5%) and 0.1% formic acid (0.1%) in methanol (95%) as mobile phases I and II, respectively, and managed to flow at 0.2 mL/min rate. The lined gradient manner elution was as follows (Table 2).

Table 2
Lined gradient manner elution for LC-MS

Time (min)	% of formic acid (0.1%) in 5% methanol (5%)	% of 0.1% formic acid (0.1%) in methanol (95%)
0.25	60	40
0.25	30	70
2	15	85
2.4	2.0	98
2.9	60	40

The column as well as autosampler container were kept at 35 ^oC as well as 4 ^oC, respectively. The electrospray input parameters for positive format ionization of RRT Impurity 1.26 and doxorubicin were: 260 °C - gas temperature; 30 psi – nebulizer pressure; 11 lt per min - gas flow; 400 °C - sheath gas temp.; 4000 V - capillary voltage; 12 lt per min - sheath gas stream; 2000 V – voltage at nozzle. At quite pressure of 2 bar, nitrogen gas being employed on the instrument as the collision gas. The collision energy for RRT Impurity 1.26 and doxorubicin fragmentation were 9 eV.

2.10. LC-MS investigation on RRT Impurity 1.26 and doxorubicin

RRT Impurity 1.26 and Doxorubicin were injected (20 µl sample) onto an Omega Luna C18 column (50 mm in length, 2.1 mm in diameter, and 1.6 m in size), and the sample was then investigated utilizing the criteria outlined in the passage under “LC-MS analysis conditions”. The ion polarity had been set to positive ion configuration for RRT Impurity 1.26 and doxorubicin mass measurement, and mass spectra for RRT Impurity 1.26 and Doxorubicin were collected in the *m/z* value scope of 50 to 1000.

3. Results and Discussion

3.1. Isolation of RRT Impurity 1.26

The impurity with RRT 1.26 was isolated using a simplified lined gradient solvent system that was addressed in the experimental portion “HPLC conditions to isolate RRT Impurity 1.26”. The RRT Impurity 1.26 was eluted using this solvent solution at around 28.010 min (Fig. 1). The separated RRT Impurity 1.26 fractions were concentrated by evaporating the acetonitrile at ambient temperature while operating under high vacuum on anequipment rotavapour. By lyophilizing the obtained aqueous RRT Impurity 1.26 solutions, the RRT Impurity 1.26 was solidified. The RRT Impurity 1.26 impurity was secluded in amounts of 20.0 mg.

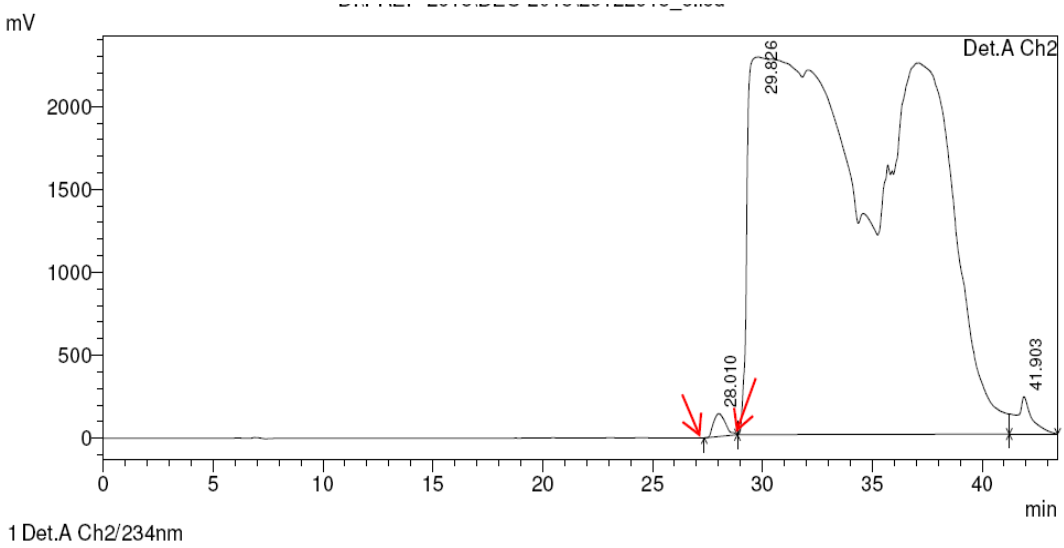


Fig. 1. RRT Impurity 1.26 chromatogram

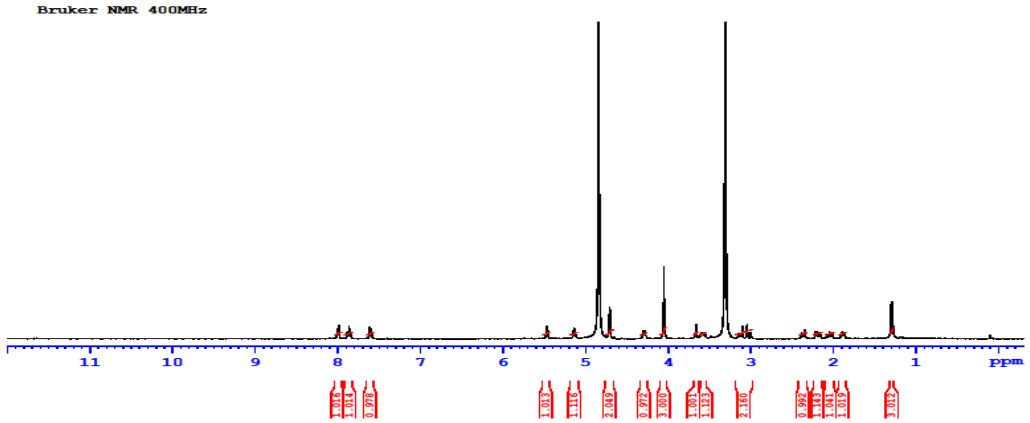


Fig. 2. 1HNMR spectra for doxorubicin API

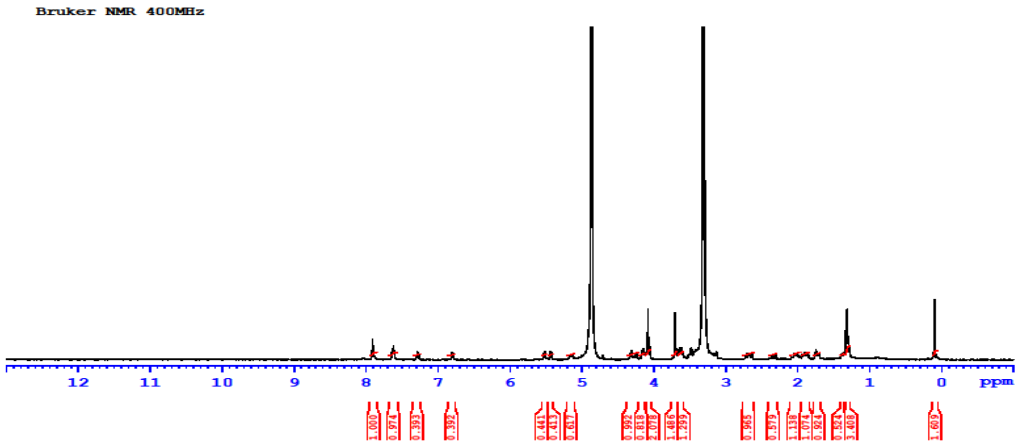


Fig. 3.[1]HNMR spectra for RRT Impurity 1.26 after 0 hr of isolation

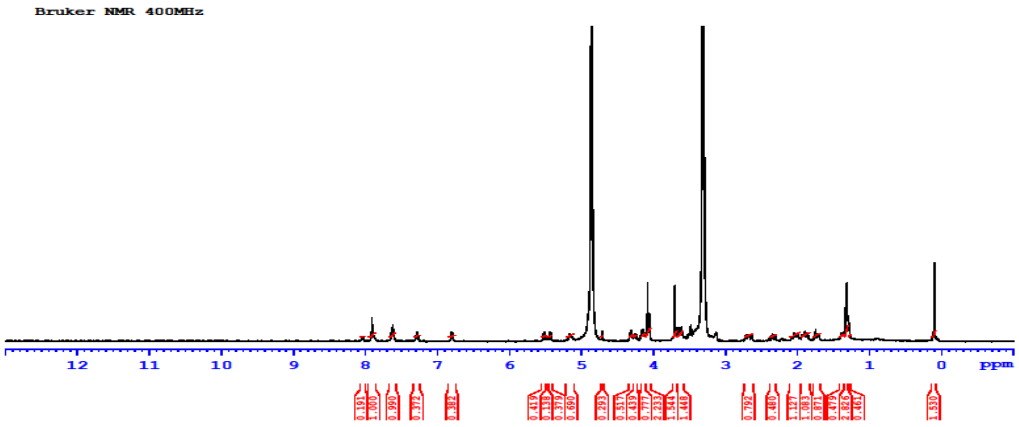


Fig. 4. [1]HNMRspectra for RRT Impurity 1.26 after one hr of isolation

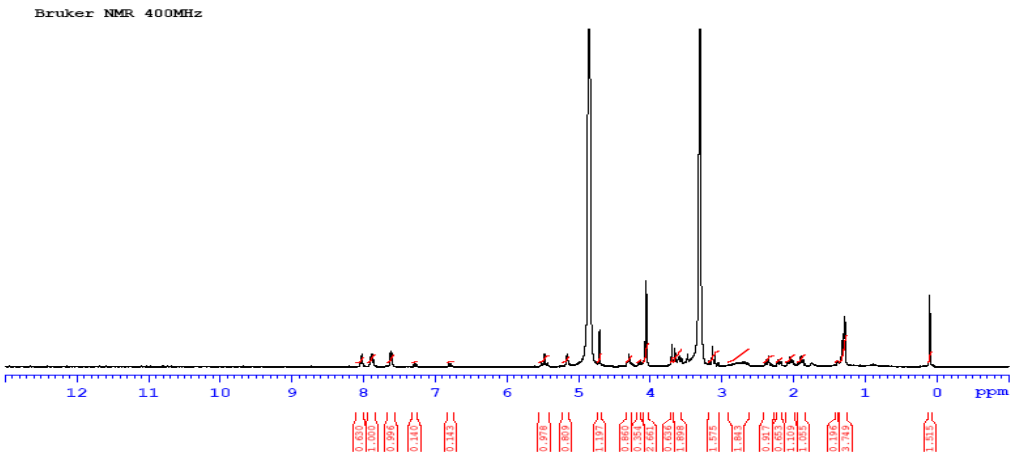


Fig. 5. [1]HNMRspectra for RRT Impurity 1.26 after fifteen hrs of isolation

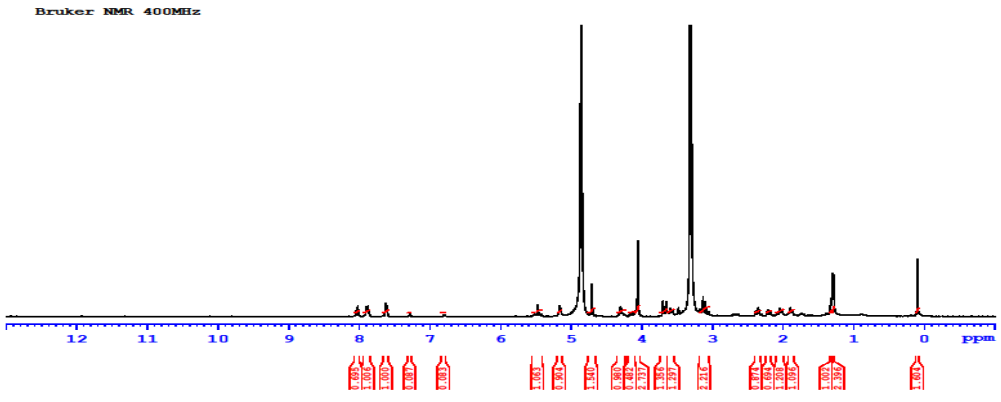


Fig. 6. [1]HNMR spectra for RRT Impurity 1.26 after twenty-four hrs of isolation

3.2. [1]H NMR investigation of RRT Impurity 1.26

The ^{1}H NMR spectra (Fig. 2) for doxorubicin API was obtained. After 0 hr (Fig. 3), 1 hr (Fig. 4), 15 hr (Fig. 5), and 24 hr (Fig. 6) of isolation, the ^{1}HNMR spectras for RRT Impurity 1.26 were obtained.

Conferring to the aforementioned NMR data, the isolated RRT Impurity 1.26 began converting into doxorubicin API before 1 hour and finished doing so after 24 hr. Doxorubicin API has 22 protons plus 7 exchangeable protons, that aren't visible in NMR since we employed the CD_3OD as NMR solvent. The isolated RRT Impurity 1.26 has 21 protons but lacks a typical peak of -CO-CH_2 at 4.7 ppm from the doxorubicin API NMR spectra, which might be transforming into an enol group (=CH-OH) with one exchangeable proton. The existence of two sets of protons, which may be isomers. The isolated RRT Impurity 1.26 is not stable to check the C13NMR and 2D NMR (COSY or HMBC) complete data.

3.3. LC-MS investigation of RRT Impurity 1.26 and doxorubicin

Doxorubicin and isolated RRT Impurity 1.26 electrospray ionization mass spectral data were acquired in positive ion format. The Fig. 7 and 8 of the mass spectrums appear to suggest a molecular ion at *m/z* 544.3 (M+H), which corresponds to the molecular weight of doxorubicin and isolated RRT Impurity 1.26, which is 543.17 a.m.u. The mass spectrum of doxorubicin and isolated RRT Impurity 1.26 are in agreement with the structure of doxorubicin (Fig. 9) and isolated RRT Impurity (Fig. 10).

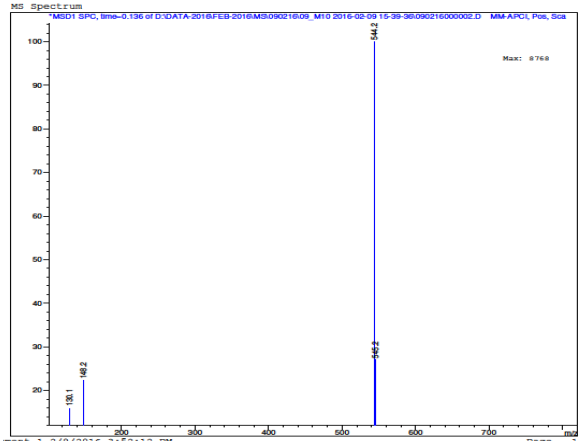


Fig. 7. Spectrum of Mass Ionization of doxorubicin

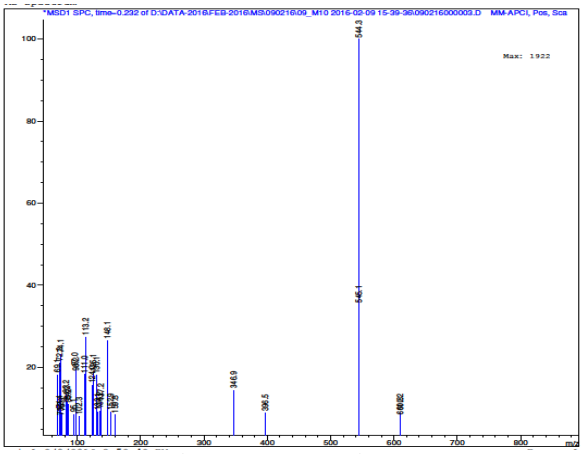
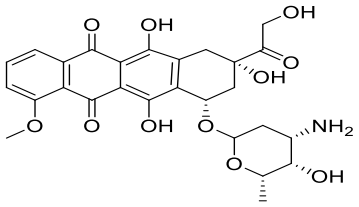
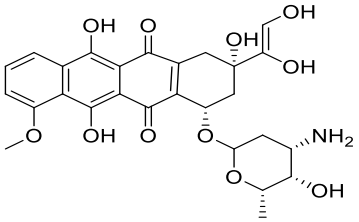


Fig. 8. Spectrum of Mass Ionization of isolated RRT Impurity 1.26



Chemical Formula: $C_{27}H_{29}NO_{11}$
Exact Mass: 543.17
Elemental Analysis: C, 59.66; H, 5.38; N, 2.58; O, 32.38

Fig. 9. Doxorubicin structure and mass using Spectrum of Mass Ionization



Chemical Formula: $C_{27}H_{29}NO_{11}$
Exact Mass: 543.17
Elemental Analysis: C, 59.66; H, 5.38; N, 2.58; O, 32.38

Fig. 10. Proposed isolated RRT Impurity 1.26 structure and mass using Spectrum of Mass Ionization

4. Conclusion

The tests, for the first time, that produced the potential structure for the unidentified RRT Impurity 1.26 have been discussed. The ^{1}HNMR detects the presence of 21 protons, and the molecular weight of the suggested unidentified RRT impurity 1.26 is likewise supported by the MS data. The potential structure was hypothesized based on data from the analysis of NMR and MS spectra. Additionally, doxorubicin API and RRT Impurity 1.26 seems to be interconverted based on NMR data, and they appear in various RTs in HPLC as the interconvertible functional groups have various polarities.

5. Conflicts of interest

The authors declares that there are no potential conflicts of interest.

6. Acknowledgements

The authors are thankful to Acharya Nagarjuna University and INTI International University, Malaysia for support and encouragement.

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