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Nuclear Magnetic Resonance, Mass Spectrometer and HPLC Based Identification and Characterization of an Unidentified Impurity in Busulfan

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KEYWORDS

Busulfan, Unknown impurity, Preparative HPLC, LC-MS characterization, NMR structure elucidation

ABSTRACT:

Introduction: Busulfan is an alkylating representative that is used as an antineoplastic /cytotoxic chemotherapeutic drug. The safety and effectiveness of busulfan are directly influenced by its quality.

Objectives: The main focus of this work was to identify and characterise an unidentified impurity that consistently eluted in HPLC analysis at a relative retention time period of 0.34 after the predominant peak, busulfan.

Methods: The unknown busulfan impurity isolation was accomplished using a Luna column C18 (length of 250 mm, identification of 50 mm, particle size of 10 µm). 10 mM ammonium acetate (solvent I) and acetonitrile (solvent II) were mixed to make the gradient mode mobile phase. LC-MS analysis of unknown RRTP 0.34 impurity isolated was accomplished using a Luna column C8 (length of 50 mm, identification of 2 mm, particle size of 3 µm). Ammonium formate (10 mM) and formic acid (0.1%) was used as mobile phase I. Acetonitrile containing 0.1% formic acid was used as solvent mobile phase II. The structure of unknown busulfan impurity was confirmed using ^{1}HNMR, ^{13}CNMR, and DEPT techniques.

Results: The new impurity at retention period near to 5.384 min was realized to be an unknown compound and we labelled it as unknown RRTP 0.34 impurity. A quantity of 20.0 mg of the unknown RRTP 0.34 impurity was extracted. The mass investigation of unknown RRTP 0.34 impurity revealed a molecular ion peak at m/z 264.2 $[M+H]^{+}$. The ^{1}H NMR of the isolated unknown RRTP 0.34 impurity reveals 14 protons, while the ^{13}C NMR reveals 7 carbons, comprising 2 methyl, 4 secondary, and 1 quaternary carbon, as confirmed by DEPT.

Conclusions: Based on obtained spectrum facts, the molecular chemical formula of unknown RRTP 0.34 impurity is $C_7H_{14}O_5S$ and the consequent structure was regarded as 4 (methylsulfonyloxy) butyl acetate.

1. Introduction

During all stages of drug formulation investigation, manufacturing, and production, the detection and structural characterization of known and unknown impurities becomes critical [1,2]. To determine the stability indicating characteristics of a formulation, information on the impurity profiling of the bulk drug ingredient employed in its manufacture process, particularly synthesis-correlated impurities against degradation products, should indeed be obtained[3]. Impurities must be identified, qualified, and quantified in order to assess the efficacy and quality of

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pharmaceutical medicinal substances and their related dosage forms.

Busulfan is indeed an antineoplastic drug which has a preferential depressive effect on the bone marrow due to its nonspecific alkylating activity during the cell cycle[4-6]. Busulfan is administered to treat chronic myelogenous leukaemia, polycythaemia vera, myelofibrosis, as well as primary thrombocythemia as a palliative therapy [7-9]. In polycythaemia vera sufferers, busulfan therapy is extended for 4 to 6 weeks. As a result, maintaining the quality of the busulfan product over its whole shelf life is critical in giving effective and secure therapy to sufferers.

This study's major goal was to isolate and then characterise an unknown impurity that regularly eluted after the principal peak (busulfan) in HPLC analysis at relative retention time period (RRTP) of 0.34. Since process-related impurities in busulfan can have a substantial influence on quality and reliability of busulfan, it is crucial to examine and regulate the impurity created during busulfan manufacture. According ICH requirements criteria, any impurity discovered at a concentration of exceeding than 0.1% percent in the busulfan material should be detected, isolated and characterised properly [10].

A significant quantity of RRTP 0.34 impurity was extracted for structural identification, and the RRTP 0.34 impurity was structurally investigated employing mass spectrometry plus NMR spectroscopy. By analysing the mass spectrometer data, the unknown RRTP 0.34 impurity was identified. This unknown RRTP 0.34 impurity has don't ever been reported previously, to the finest of our awareness.

2. Materials and Methods

2.1. Chemicals

A Milli-Q plus water treatment equipment (Millipore, USA) was used to make deionized water. Acetonitrile (LC grade), tetrahydrofuran (AR grade), ammonium acetate (AR grade), Ammonium formate (AR grade) and formic acid (AR grade) were obtained from “Merck Limited” (India).

2.2. HPLC analysis – instrumentation and conditions

HPLC analysis of busulfan was accomplished using a YMC ODS pack A column (length of 150 mm, identification of 4.6 mm, particle size of 5 µm) on a Water's alliance 2690 separation module (Water's company, USA) connected to a 2487 UV detector (Water's company, USA). Acetonitrile, water, and tetrahydrofuran were mixed in a 65:30:5 (*v/v/v*) ratio to make the mobile phase. The experiment was performed in isocratic mode employing a flow velocity of 1.0 ml per minute, a sensor wavelength of 210 nm, and a column adjusted temperature of 25°C throughout the experimentation. For a busulfan sample quantity of 150 µg/ml produced in mobile phase, the injecting volume for analysis was 20 µl.

2.3. Preparative HPLC - instrumentation and conditions

The unknown RRTP 0.34 impurity isolation was accomplished using a Luna column C18 (length of 250 mm, identification of 50 mm, particle size of 10 µm) on a Shimadzu LC-8A separation module (Shimadzu, Japan) connected to a UV detector (Shimadzu, Japan). 10 mM ammonium acetate (solvent I) and acetonitrile(solvent II) were mixed to make the mobile phase.The gradient mode programme used was: 0 min: 55% solvent I and 45% solvent II; 10 min: 55% solvent I and 45% solvent II; 20 min: 35% solvent I and 65% solvent II; 25 min: 35% solvent I and 65% solvent II; 30 min: 25% solvent I and 75% solvent II. The experiment was performed in gradient mode employing a flow velocity of 60 ml per minute, a sensor wavelength of 210 nm, and a column adjusted temperature of 25 °C throughout the experimentation. The injecting volume for analysis was 6 ml.For every injection, the impurity portions with a retention period of 32 to 39 min were collected into several volumetric flasks. Applying the above-mentioned conditions, 20.0 mg of the unknown RRTP 0.34 impurity was extracted.

2.4. Mass spectrometry analysis - instrumentation and conditions

For isolated sample indentation, an Agilent 6120 precise single quadrupole LC/MS equipment with analyst version software 1.6.2 and an ESI source for reliable mass measurements was employed. For mass measurement, the ion polarity was tuned to positive ion type and mass spectra was retrieved in the m/z value of 50 -1000 range. The capillary voltage was kept at 3500 V throughout the experiment. The curtain gas remained

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at 20 psi pressure, the nebulizer gas flow was with 54 psi pressure.

LC-MS analysis of unknown RRTP 0.34 impurity isolated was accomplished using a Luna column C8 (length of 50 mm, identification of 2 mm, particle size of 3 µm). Ammonium formate (10 mM) and formic acid (0.1%) was used as solvent I. acetonitrile containing 0.1% formic acid was used as solvent II. The solvent I and II were mixed to make the mobile phase. The gradient mode programme used was: 0 to 0.5 min: 80% solvent I and 20% solvent II; 0.5 to 1.0 min: 20% solvent I and 80% solvent II, maintained at this rate for 0.5 min; 1.5 to 3.0 min: 80% solvent I and 20% solvent II. The experiment was performed in gradient mode employing a flow velocity of 0.7ml per minute. The injecting volume for analysis was 20 µl.

2.5. NMR Analysis - instrumentation and conditions

NMR spectrum of unknown RRTP 0.34 impurity was recorded with400 MHz NMR spectrometer (Bruker, Avance III, Switzerland). Samples were made dissolved in deuterated $CDCl_3$ at 5 mg/mL concentration. The peak at 7.26 ppm was utilised as a reference for the protons chemical shifts since it corresponded to remaining chloroform chosen for dilution. The ^{1}H NMR spectra of unknown RRTP 0.34 impurity were obtained by performing 16 scans and recording them with a 1 s pulse repeating time by employing an 30° flipping angle. ^{13}C NMR spectra were obtained by doing 1024 scans and recording them with voltage gated decoupling at a 30° flipping angle with a 2 s repetition period. The chemical shifts of ^{13}C NMR, 1H NMR and DEPT(Distortionless enhancement via polarization transfer) were measured on a delta magnitude in ppm with respect to $CDCl_3$. All spectra were taken with the sample spinning at a rate of 20 Hz.

3. Results

3.1. Detection of Impurity by HPLC

The HPLC method was used to examine busulfan drug samples, as specified in the section “HPLC analysis – instrumentation and conditions”. As can be seen in Figure 1, analysis indicated the occurrence of a new impurity at retention period 5.384 min with RRTP of 0.34.

3.2. Isolation of unknown RRTP 0.34 impurity

Isolation of unknown RRTP 0.34 impurity was done using conditions as specified in the section “Preparative HPLC - instrumentation and conditions”. The impurity portion fractions from several infusions were obtained and then combined. The chromatogram obtained while isolation was included (Figure 2). These fractions were concentrated employing rotavapor in a high-vacuum environment. The unknown RRTP 0.34 impurity was solidified by lyophilization of the aqueous unknown RRTP 0.34 impurity solutions.

The extracted unknown RRTP 0.34 impurity was examined as specified in section “HPLC analysis – instrumentation and conditions” to ensure its purity. The RRTP of isolated impurity was also confirmed through comparison with busulfan sample having the unknown RRTP 0.34 impurity. Figure 3 and 4 show the final results.

3.3. Identification of unknown RRTP 0.34 impurity by mass spectrometer

The method as specified in section “Mass spectrometer analysis - instrumentation and conditions” was employed to identify unknown RRTP 0.34 impurity. The mass investigation of unknown RRTP 0.34 impurity revealed a molecular ion peak at m/z 264.2 $[M+H]^{+}$(Figure 5), and the potential structure, chemical name, formula, mass, and molecular weight for the unknown RRTP 0.34 impurity was hypothesised relying on this mass estimate, as illustrated in Figure 6.

3.4. Unknown RRTP 0.34 impurity structure confirmation via NMR analysis

The NMR signals for the unknown RRTP 0.34 impurity were assigned employing ^{1}H, ^{13}C, and DEPT spectroscopy. Tables 1 and 2 list the relevant data, whereas Figures 10,11, and 12 depict the spectrum.

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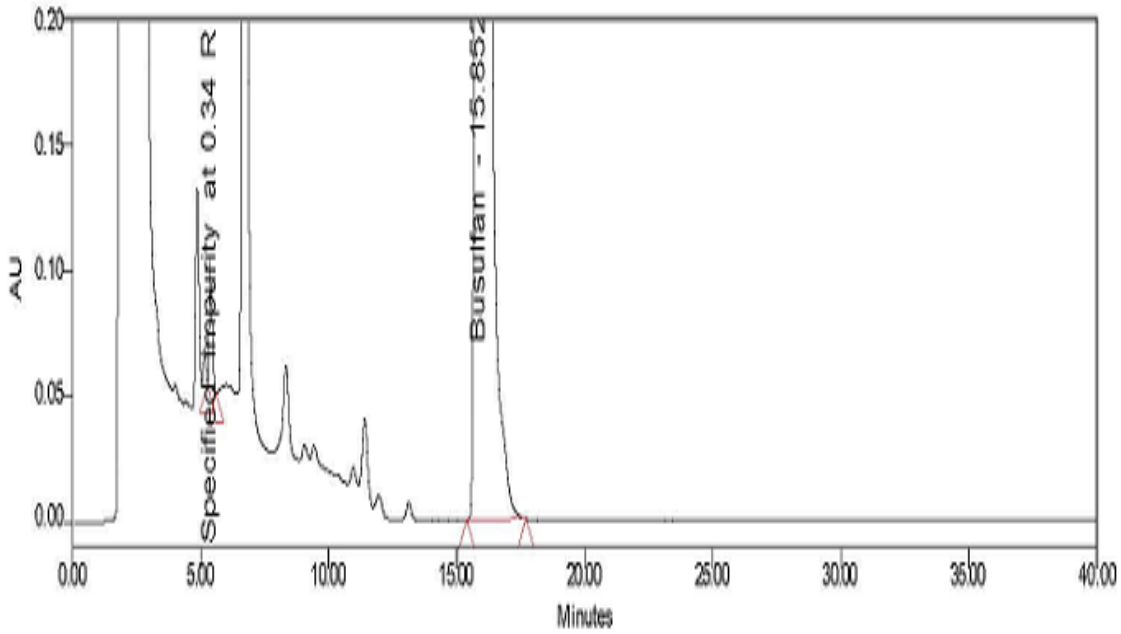

Fig. 1. Chromatogram showing peaks of busulfan and unknown RRTP 0.34 impurity

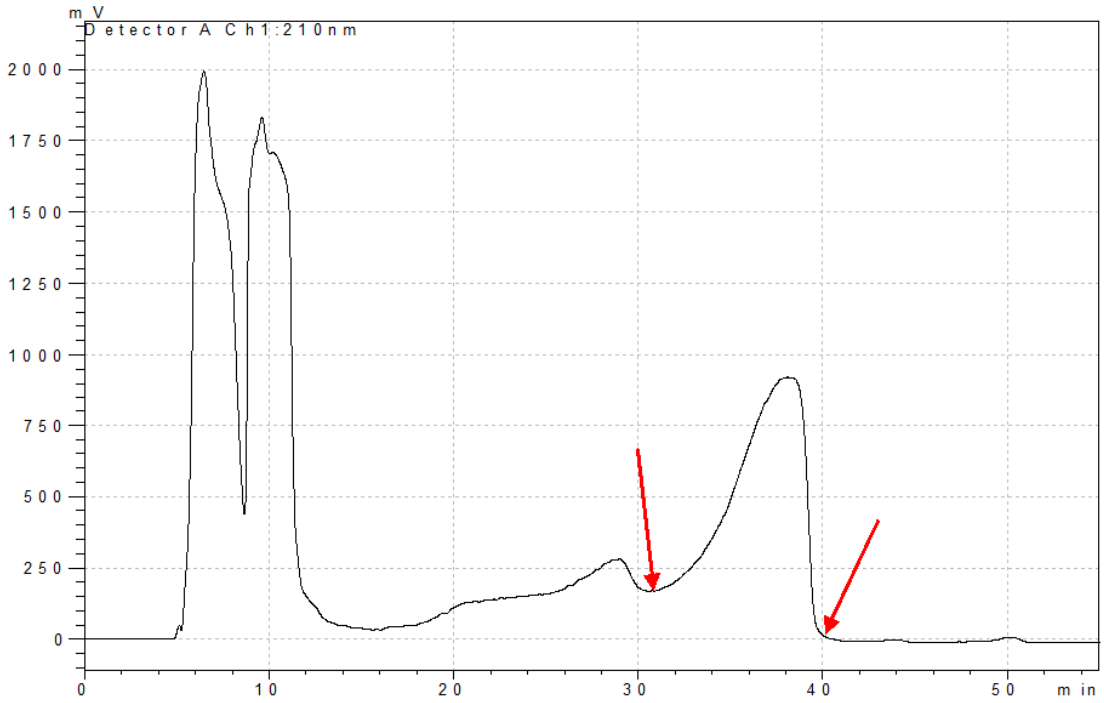

Fig. 2. Preparative chromatogram of Busulfan unknown RRTP 0.34 impurity

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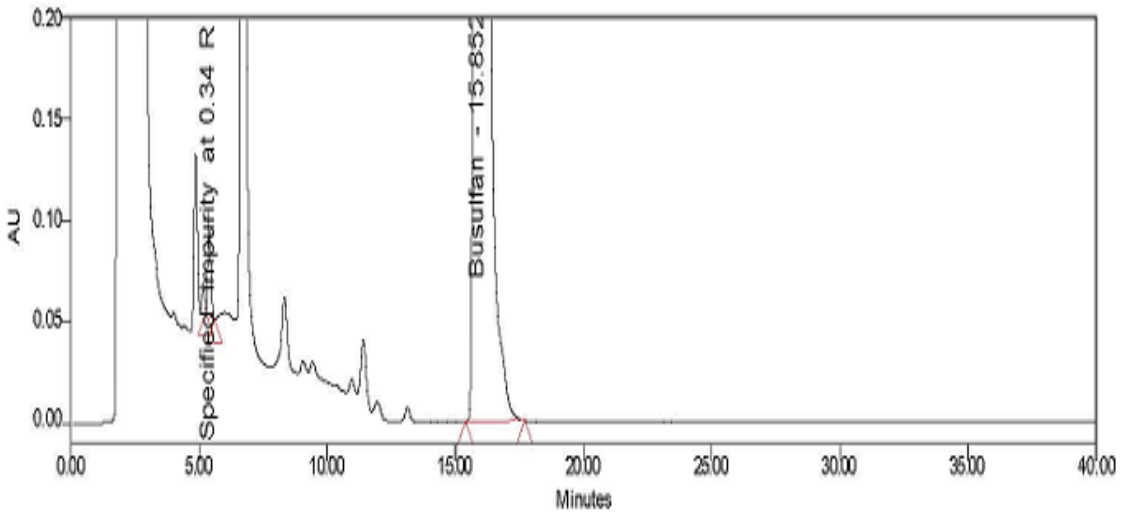

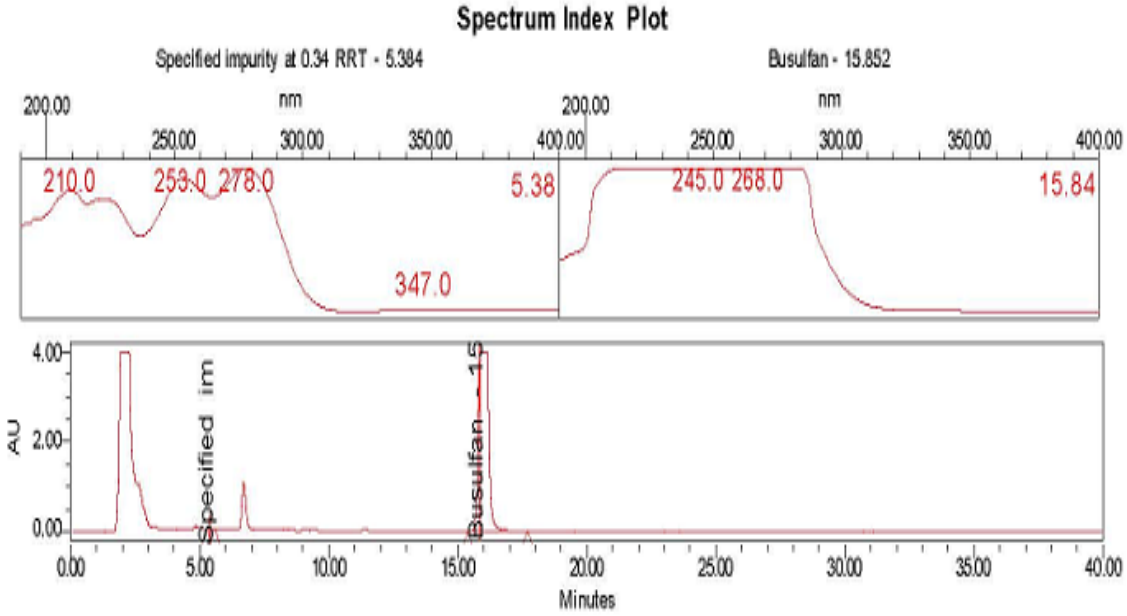

Peak Results

	Name	RT	Area	% Area	Purity1 Angle	Purity1 Threshold	Purity1 Flag
1	Specified impurity at 0.34 RRT	5.384	349362	0.30	1.036	3.864	No
2	Busulfan	15.852	114655627	99.70	9.279	21.794	No

Fig. 3. RRTP confirmation and peak purity chromatogram of busulfan with unknown RRTP 0.34 impurity

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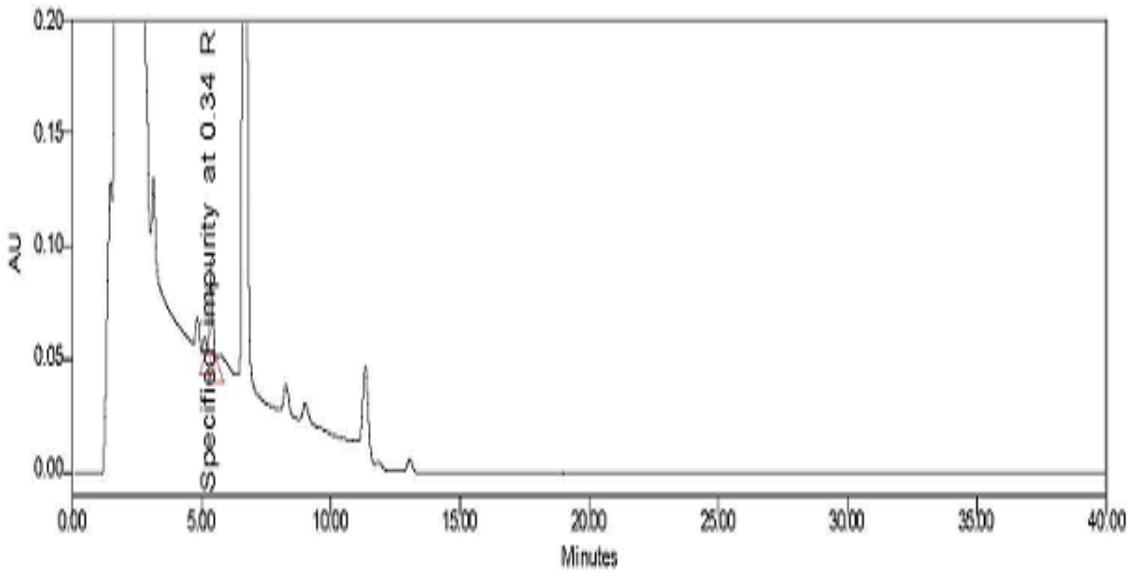

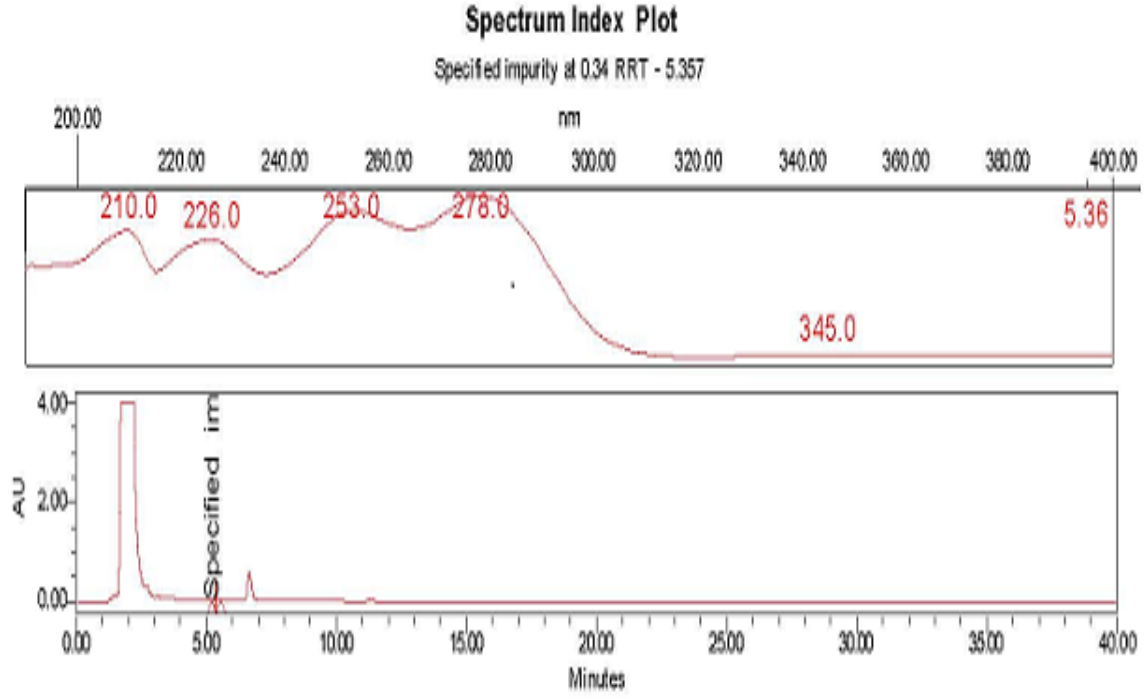

Peak Results

	Name	RT	Area	% Area	Purityl Angle	Purityl Threshold	Purityl Flag
1	Specified impurity at 0.34 RRT	5.357	279038	100.00	0.624	3.832	No

Fig.4. RRTP confirmation and peak purity chromatogram of isolated unknown RRTP 0.34 impurity

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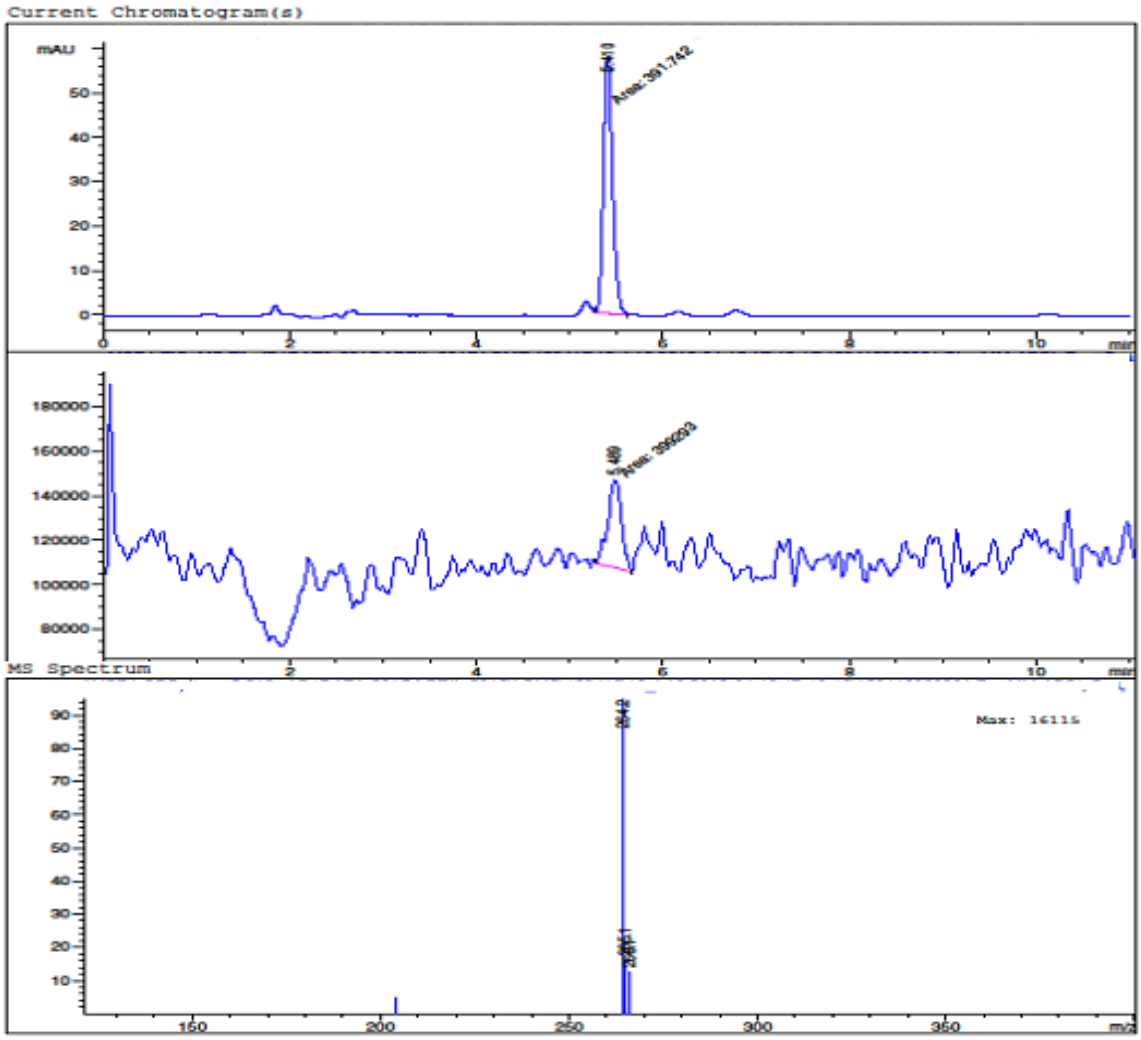
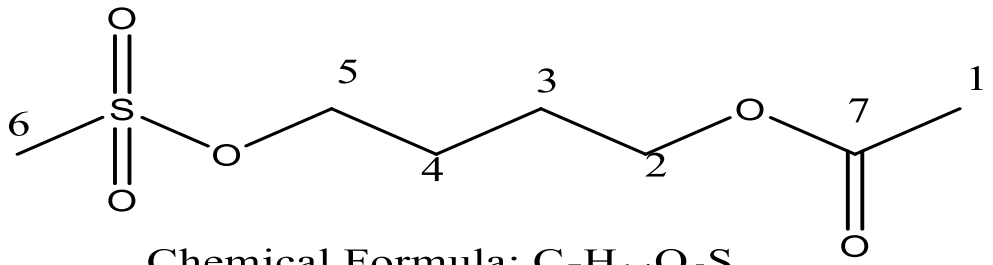


Fig. 5. Mass ionization spectrum of unknown RRTP 0.34 impurity isolated through adopting preparative HPLC process



Chemical Formula: $C_7H_{14}O_5S$
Exact Mass: 210.06
Molecular Weight: 210.25

4-(methylsulfonyloxy)butyl acetate

Fig. 6. Proposed structure of unknown RRTP 0.34 impurity

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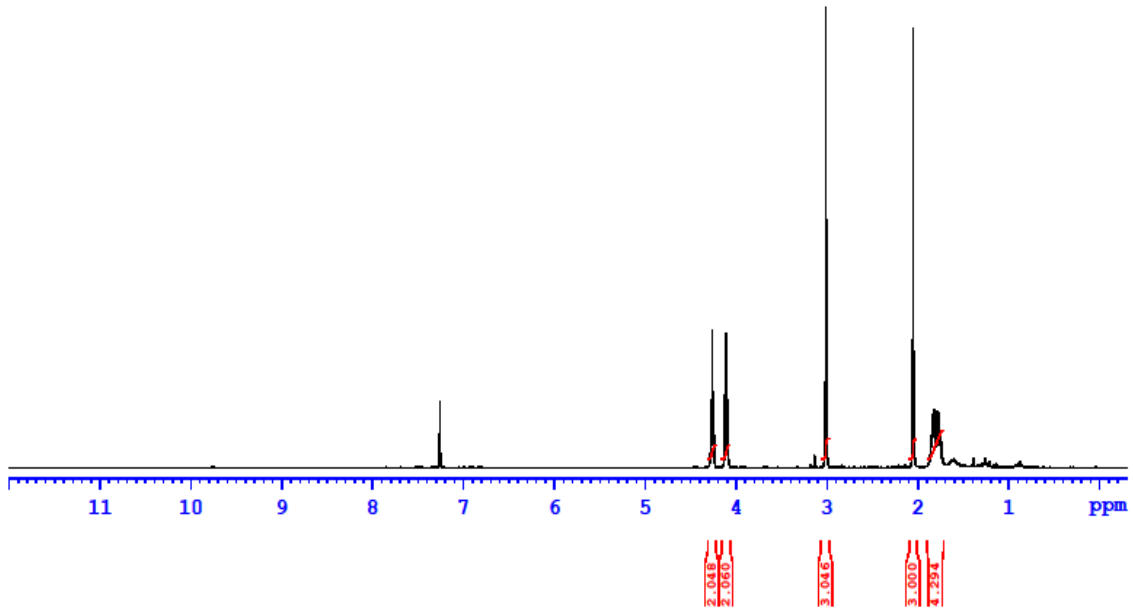

Fig. 7. [1]H spectra of unknown RRTP 0.34 impurity

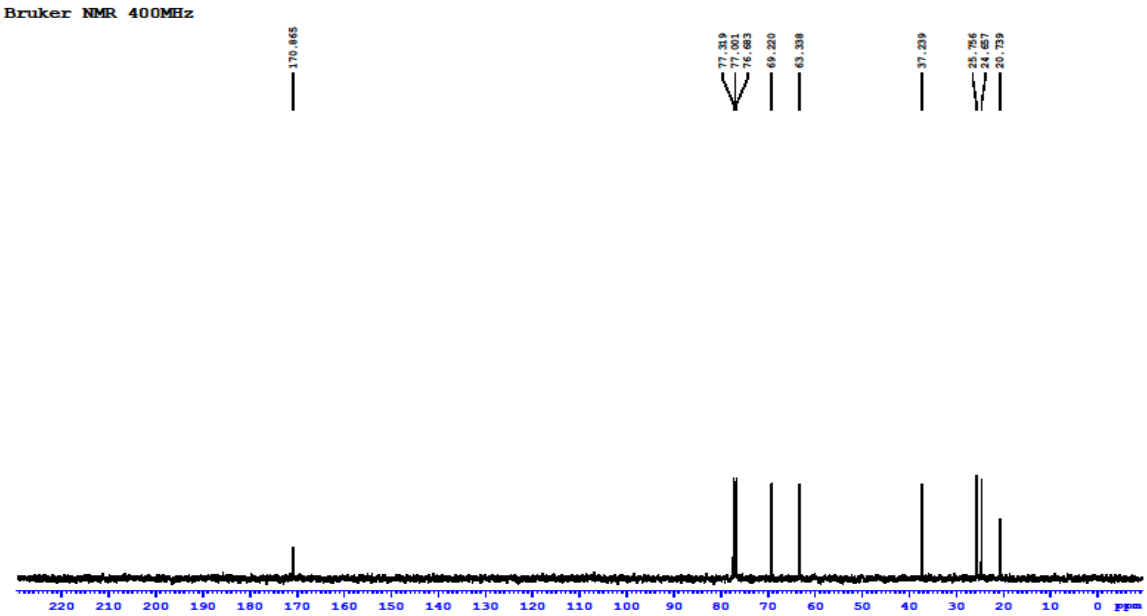

Fig. 8. [13]C spectra of unknown RRTP 0.34 impurity

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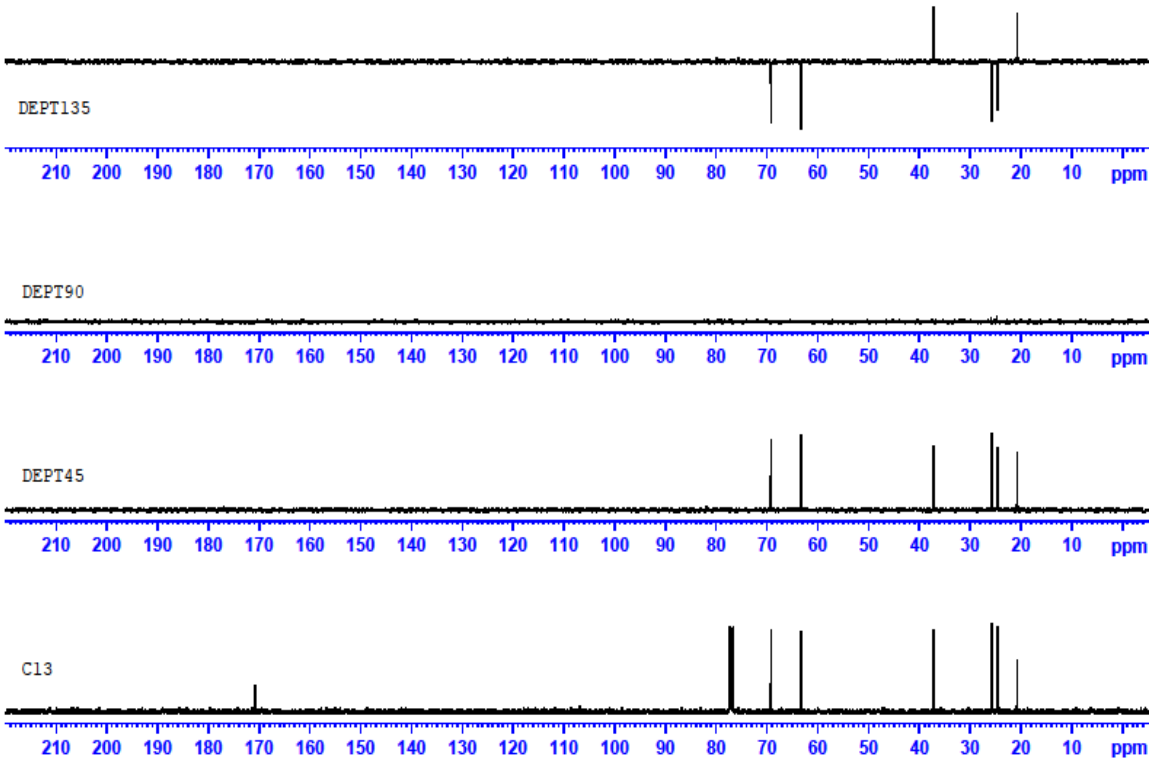

Fig. 9. DEPT spectra of unknown RRTP 0.34 impurity

Table 1: ^{1}H NMR Peak Assignments for unknown RRTP 0.34 impurity

Proton	Chemical shift, ppm	No. of protons	Multiplicity
H1	2.05	3	s
H2	4.24-4.27	2	t; $3J_{HH}$ = 5.9 HZ
H3	1.85-1.80	2	m
H4	1.80-1.75	2	m
H5	4.12-4.09	2	t; $3J_{HH}$ = 5.9 HZ
H6	3.01	3	s
Total number of protons identified= 14			
$CDCl_3$	7.26		

s = singlet, t = triplet, m = multiplet

Table 2: ^{13}C NMR Peak Assignments for 'C'

Carbon #	Chemical Shift (δ), ppm
C1	20.73
C2	63.33
C3	24.65
C4	25.75
C5	69.22
C6	37.23
C7	17.86
$CDCl_3$	77.00

4. Discussion

In analyses of busulfan drug samples, a new impurity was discovered at values of greater than 0.1%, resulting in an outside of specification result (In other words, the unknown impurity would never exceed 0.10 percent). This new impurity at retention period near to 5.384 min was realized to be an unknown compound and labelled it as unknown RRTP 0.34 impurity. 20.0 mg of the unknown RRTP 0.34 impurity was extracted. The purity of an unknown RRTP 0.34 impurity was confirmed by the peak purity and threshold values. In both the busulfan sample and the isolated sample, the RRTP value of unknown RRTP 0.34 impurity was the same. Based on obtained spectrum facts and relying on the mass estimates, the potential structure, chemical name, formula, mass, molecular weight and molecular chemical formula of unknown RRTP 0.34 impurity is $C_7H_{14}O_5S$ and the consequent structure was regarded as4 (methylsulfonyloxy) butyl acetate.

Journal of Chemical Health Risks

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JCHR (2024) 14(6), 1160-1169 | ISSN:2251-6727

5. Conclusion

The experiments that led to the likely proposed structure for the unknown RRTP 0.34 impurity were discussed in this article. The ^{1}H NMR of the isolated unknown RRTP 0.34 impurity reveals 14 protons, while the ^{13}C NMR reveals 7 carbons, comprising 2 methyl, 4 secondary, and 1 quaternary carbon, as confirmed by DEPT. The potential structure of unknown RRTP 0.34 impurity was suggested based on comparison of NMR spectrum information data. In HPLC, it was likewise proven to be 0.34 RRTP when compared to the busulfan peak.

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