Available online at www.derpharmachemica.com

ISSN 0975-413X
CODEN (USA): PCHHAX

Der Pharma Chemica, 2017, 9(20):16-24
(http://www.derpharmachemica.com/archive.html)

Identification, Isolation and Characterization of Process Related Impurity in Isosulfanblue

Chandra Sekhara Rao Nethinti[1,2*], Sreenivasulu Boju[1], Raghu Babu Korupolu[2], Annapurna Nowduri[2], Uttam Kumar Ray[1]

[1]Chemical Research and Development Department, Aurobindo Pharma Ltd, Survey No: 71 & 72, Indrakaran, Sangareddy, Medak, Hyderabad-502329, Telangana, India
[2]Department of Engineering Chemistry, A.U. College of Engineering (A), Andhra University, Visakhapatnam-530 003, Andhra Pradesh, India

ABSTRACT

A new unknown impurity of isosulfanblue formed during the synthesis of isosulfanblue, and the level of the impurity was found at below 0.10%. This impurity was identified by Liquid Chromatography-Mass Spectrometry (LC-MS) and characterized by Proton Nuclear Magnetic Resonance (^{1}H-NMR), Carbon-13 Nuclear Magnetic Resonance (^{13}C-NMR), MS and Fourier-Transform Infrared Spectroscopy (FTIR). Based on the spectral data, the impurity was named as, N-4-[bis[4-(diethyl amino)phenyl] hydroxymethyl]-benzene-2,5-disulphonic acid. This impurity was isolated by preparative High-performance Liquid Chromatography (HPLC) and co-injected into HPLC to confirm the retention time. Isolation, structural elucidation of the impurity and its possible mechanism of formation has been discussed. To the best our knowledge, the formation, identification, isolation, characterization of the observed new impurity was not mentioned in the literature till date.

Keywords: Isosulfanblue, Process related impurity, Identification, Isolation, Hydroxy isosulfanblue

INTRODUCTION

Isosulfanblue, chemically known as N-[4-[[4-(Diethyl amino) phenyl](2,5-Disulfophenyl)methylene]-2,5-cyclohexadien-1-ylidene]ethanaminium hydroxide, inner salt, sodium salt. Isosulfanblue is a triarylmethane dye used as a contrast agent for the delineation of lymphatic vessels and is particularly useful as a cancer diagnostic agent [1]. It has been used with increasing frequency sentinel lymph nodes in breast cancer patients. Isosulfanblue guided surgical removal of cancerous tissue has been on the rise as it is cost effective and safer to use than technetium 99M radioisotope-labeled sulfur colloid [2-6]. Isosulfanblue dye was proved useful in identifying the main abdominal and thoracic lymphatic channels and sites of lymphatic disruption [7]. Several methods have been reported in the literature for the synthesis of isosulfanblue [8,9]. These also include synthesis from isoleucoacid (2-(bis(4-(diethyl amino)phenyl)-methyl)-benzene-1,4-disulfonic acid) as per the scheme given in Figure 1. One impurity was observed during the oxidation of isoleucoacid using MnO_2 [10,11] in water, along with the known impurities. A few analytical methods have been reported in the literature for the determination of isosulfanblue [12] including studies, related to its degradation and its process related impurities [13,14]. The High Performance Liquid Chromatography (HPLC) analysis of isosulfanblue sample has been performed. As per regulatory requirement, potential process and degradation related impurities are critical for the safety assessment during manufacturing process. It is mandatory to identify and characterize the impurities in the pharmaceutical products [15], if they are present above the accepted limit of 0.05%.

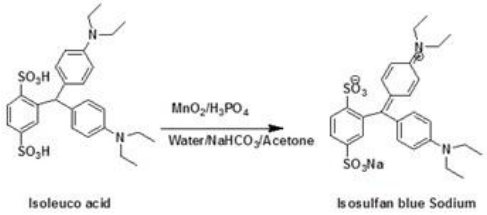


Figure 1: Reaction scheme for the synthesis of isosulfanblue blue

MATERIALS AND METHODS

Chemicals, reagents and samples

The investigated samples of isosulfanblue and known impurities were prepared in APL research center (A division of Aurobindo Pharma Limited, Hyderabad, India). The following reagents were used for analysis are ortho phosphoric acid (GR Grade), acetonitrile (HPLC Grade), water (Milli-Q grade) and formic acid (GR grade).

High performance liquid chromatography (HPLC)

Chromatographic separations were performed on HPLC system with Waters Alliance 2695 separation module equipped with 2487 dual absorbance detector with Empower pro data handling system (Waters Corporation, Milford, MA 01757, USA). The analysis was carried out on Xterra C18, 250 mm long, 4.6 mm internal diameter and 5 µm particle size columns. Mobile phase A was LC-MS compatible, volatile buffer consists Orthophosphoric Acid (OPA) in 1000 ml of water. Mobile phase B was Acetonitrile, prepared by mixing solutions A and B in the ratio of 80:20 v/v. Injection volume was 20 ml, flow rate was 1.0 ml/min and column oven temperature was 35°C. UV detection was carried out at 220 nm. Data acquisition time was 40 min. Pump was run in a gradient mode and the program was as follows: Time (min)/A v/v:B v/v; T0.01/95:0.5, T25/70:30, T40/95:05, T50/95:05.

Mass spectroscopy

The MS consisted of Xevo G2Q-TOF HRMS spectrometer (Waters micro mass, Manchester, UK) operated with Mass Lynx (Version 4.1) software. The fragmentation profile of the samples was established by carrying out MS/TDF studies in negative Electro Spray Ionization (ESI) mode. 3000 v, cone voltage 25 v, source temperature 120°C, desolation temperature 300°C, ion energy 1.0 v and collision energy 8 v. The samples were directly infused using a syringe at a concentration of 1 mg/ml in methanol.

Nuclear magnetic resonance (NMR) spectroscopy

The ^{1}H-NMR experiments (^{1}H-NMR, ^{13}C-NMR) were performed on a 500 MHz system by Agilent Technologies using deuterated water (D_2O) as a solvent and tetramethylsilane (TMS) as an internal standard at 25°C. The operating frequencies were 500.13 MHz and 125.4748 MHz.

Fourier transform infra-red (FTIR) spectroscopy

FTIR spectra were recorded in KBr Pellet on one (Pertain Elmer, Massachusetts, USA).

HPLC preparative

A preparative HPLC system equipped with Shimadzu consists of LC8A VP pumps with premixer assembly and SPD-10A VP spectrophotometric UV-Visible detector. The data was collected and processed using Shimadzu software. A stainless steel column 500 mm long, 30 mm internal diameter filled with octadecylsilane (C18) chemically bonded to pour silica particle size of 10 µ (use Hyperprep HS C18, 10 µ, 500 × 30 mm) Make: Thermo or Purtas Prep, 10 µ, 500 × 30 mm, Make: Chroma chemie. These are employed for loading the sample. An analytical method was developed in isocratic mode separately to resolve the impurity, followed by scaling up the same method for preparative HPLC to collect the required impurity fractions. The mobile phase consists of 1.5 v/v formic acid and Milli-Q water and Acetonitrile in the ratio of 1.5 v/v (80:20). The flow rate was set at ~ 30 ml/min. Detection was carried out at 220 nm. Approximately 80 mg/ml of sample was prepared using sample diluents. The sample diluents were Milli-Q water.

Impurity sample preparation

Prepared by dissolving isosulfanblue and formic acid in water and stirred for 7 days at room temperature and forming the impurity was ~ 12%.

Isolation of impurity by preparative HPLC

A preparative HPLC system, discussed under section 2.6 was used for isolating the unknown impurity eluted at about 15 min. Fractions were collected and concentrated under reduced pressure using rotavapor to obtain a crystalline solid. Purity has been checked by HPLC, which was found to be 100%, and was characterized by ^{1}H-NMR, ^{13}C-NMR, Mass and FTIR experiments (Figure 2).

RESULTS AND DISCUSSION

Detection of impurity

Isosulfanblue was analyzed by HPLC method described in section 2.2. The analysis revealed the presence of a unknown peak marked as impurity at Relative Retention Time (RRT) of 0.29 in the chromatogram. The chemical structure of impurity was given in Figure 3. The potential known impurity and isolated impurity were co-injected with isosulfanblue to confirm their relative retention times. All the impurities were well resolved from isosulfanblue peak. The representative resolution mixture chromatogram is shown in Figure 4.

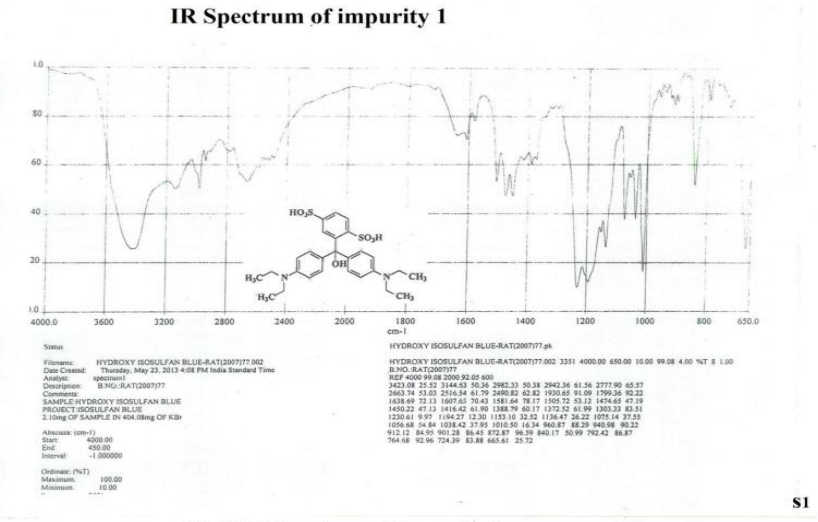
Structural elucidation of impurity

The molecular ion peak of m/z 562 [M-H] by LC-Ms analysis indicated mass of 561, which is 16 amu greater than isosulfanblue, indicates that the addition of one hydroxy group into Isosulfanblue. The major molecular fragment peaks appearing at 158, 238, 546, 466 and 562 resembled the probable fragmentation pattern of impurity (Table 1).

The ^{1}H-NMR spectrum of the new impurity showed one signal corresponding to the aromatic protons at 7.47-7.56 ppm (8H, m) but in isosulfanblue sodium ^{1}H-NMR showed two signals at 6.85 ppm (4H, d) and at 7.28 ppm (4H, d) respectively. This change in the delta value of the aromatic protons is due to the replacement of alkenic bond with hydroxy group.

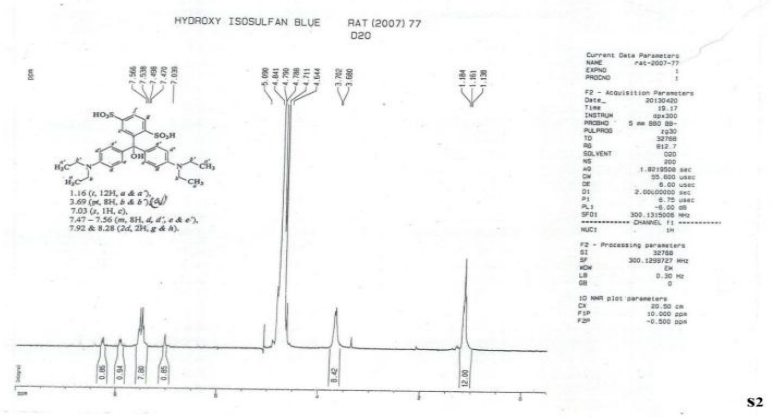
In IR (KBr, cm^{-1}) spectrum has shown a broad peak at a region of 3200-3600 cm^{-1}, which conform the presence of hydroxy group in a isolated impurity. However no such type of broad peak was observed in isosulfanblue.

IR Spectrum of impurity 1



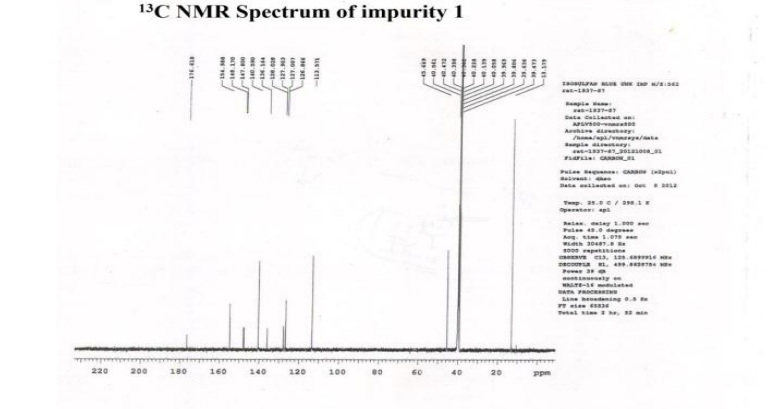
S1

^{1}H NMR Spectrum of impurity 1



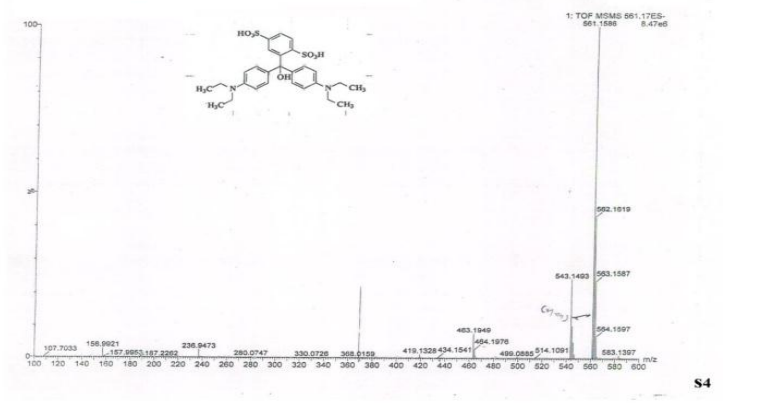
S2

^{13}C NMR Spectrum of impurity 1



S3

MASS Spectrum of impurity 1



S4

Figure 2: Characterization of impurity 1 (IR, ^{1}H-NMR, ^{13}C-NMR, mass spectrometry)

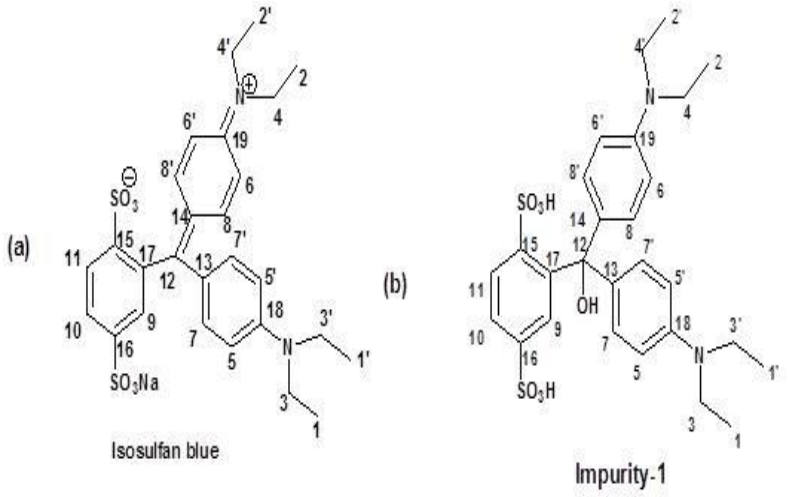


Figure 3: Chemical structure of isosulfanblue blue and its process related impurity, (a) Isosulfanblue, (b) Impurity-1

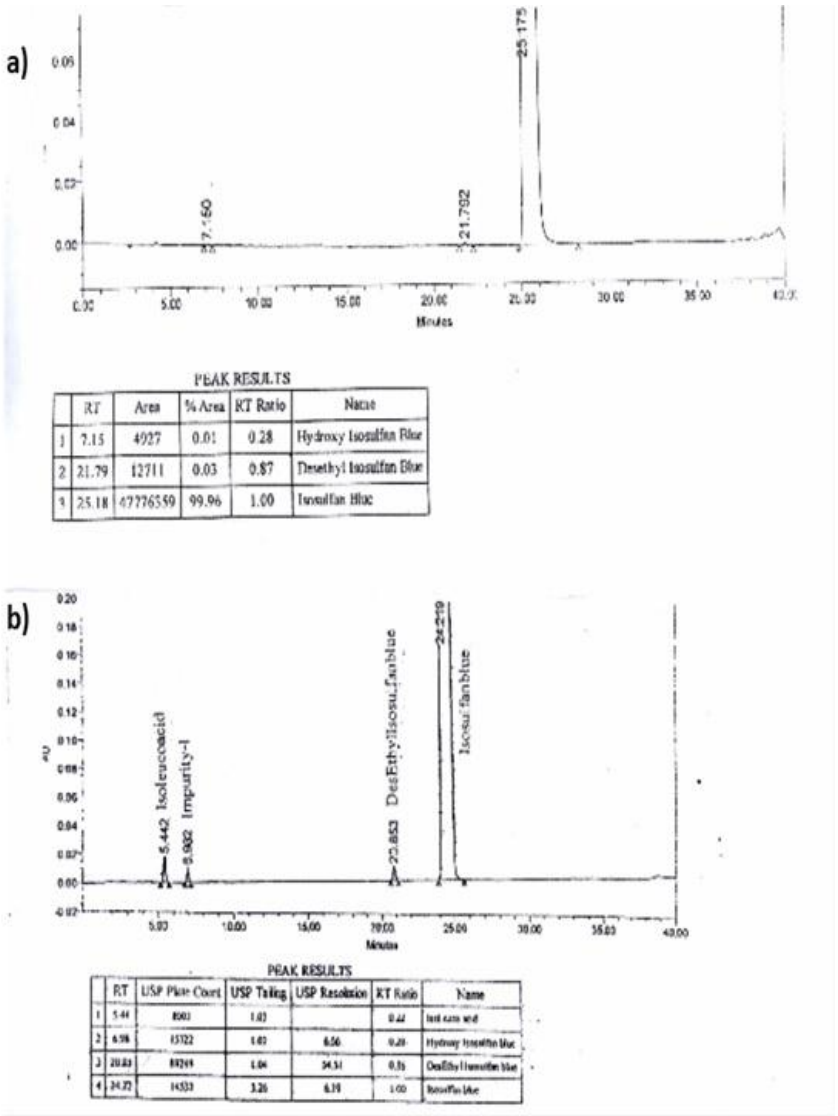
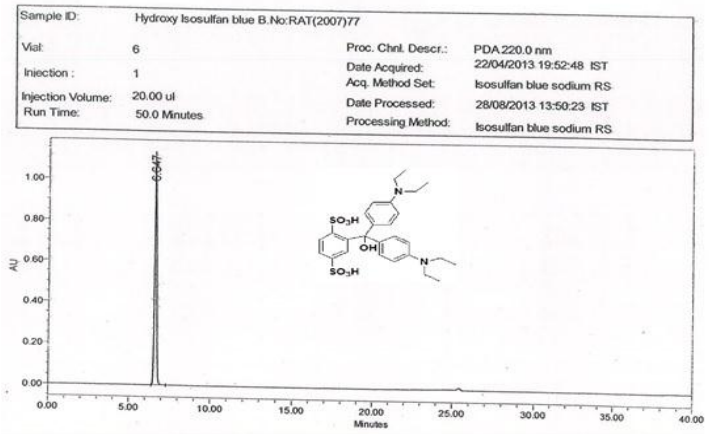


Figure 4: HPLC chromatogram of (a) Isosulfanblue and its impurities, (b) Isosulfanblue sample spiked with impurities

HPLC Chromatogram of impurity 1

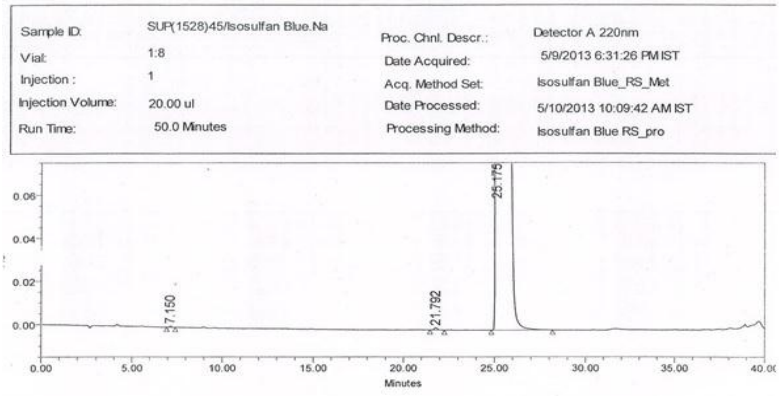


PEAK RESULTS

	RT	Area (µV*sec)	Name
1	6.65	10257711	Hydroxy Isosulfan blue

S9

HPLC Chromatogram of isosulfanblue

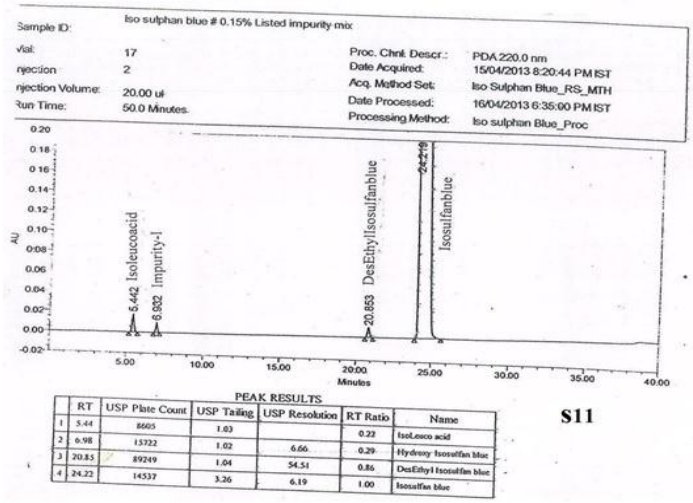


PEAK RESULTS

	RT	Area	% Area	RT Ratio	Name
1	7.15	4927	0.01	0.28	Hydroxy Isosulfan Blue
2	21.79	12711	0.03	0.87	Desethyl Isosulfan Blue
3	25.18	47776359	99.96	1.00	Isosulfan Blue

S10

HPLC chromatogram of Impurity mixture



PEAK RESULTS

	RT	USP Plate Count	USP Tailing	USP Resolution	RT Ratio	Name
1	5.44	8605	1.03		0.22	IsoLeuco acid
2	6.98	13722	1.02	6.66	0.29	Hydroxy Isosulfan blue
3	20.85	89249	1.04	54.51	0.86	DesEthyl Isosulfan blue
4	24.22	14537	3.26	6.19	1.00	Isosulfan blue

S11

Figure 5: HPLC chromatogram of impurity-1, isosulfan blue and impurity mixture

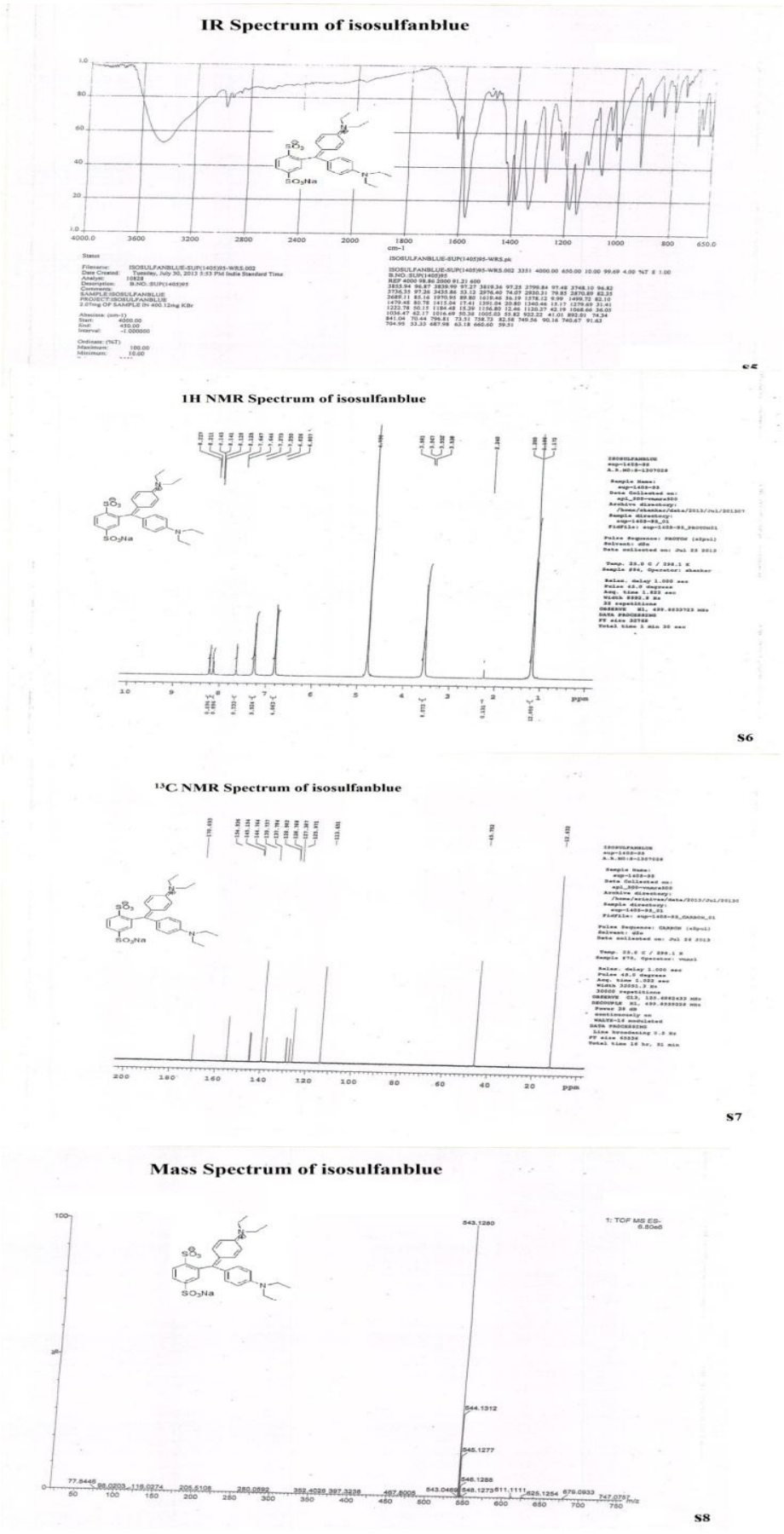


Figure 6: Characterization of isosulfanblue (IR, ^{1}H-NMR, ^{13}C-NMR, mass spectrometry)

The ^{13}C-NMR spectrum of the new impurity, benzyl carbon showed signal at 64 ppm, whereas in isosulfanblue sodium the benzyl carbon showed signal at 126.86. This shift of signal to the up field is due to the replacement of alkenic bond with hydroxyl group in the impurity.

In the isolated impurity two signals were observed for quaternary carbons (13, 12). 126.86 ppm for C_{13}, and at 64 ppm for C_{12} (Table 2). But in isosulfanblue these carbons showed one signal at 125.94 ppm for C_{12}. Based on the above analytical results it is concluded that the isolated impurity is the hydroxy isosulfanblue, and formed during the oxidation of isosulfanblue in formic acid media (Figures 5 and 6).

Table 1: Fragmentation pattern of impurity-1(m/z [(M-H)])

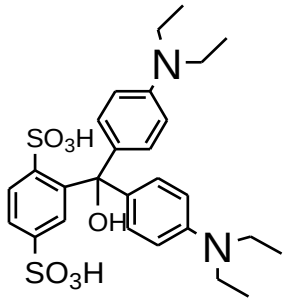
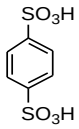
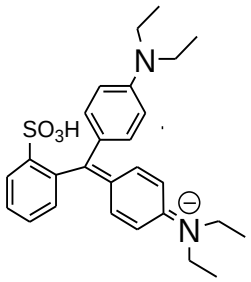
Fragment ID	Fragment structure	Theoretical mass (m/z)	Accurate mass by MS/MS/Q-TOF(m/z)[(M-H)]	Mass error (ppm)
Impurity-1		562.1807	561.1586	4.14
A		237.9605	236.9473	1.32
B		465.2	464.1976	0.24
C		158.0037	156.9921	1.16

Table 2: Comparative ^{1}H, ^{13}C (proton decoupled) NMR assignments for isosulfanblue blue and impurity-I

Position	Isosulfanblue blue		Impurity	
	^{1}H (ppm)/^{1}H/Multiplicity (J, H_Z)	^{13}C (Chemical shift in ppm) (J, H_Z)	^{1}H (ppm)/^{1}H/Multiplicity (J, H_Z)	^{13}C (Chemical shift in ppm) (J, H_Z)
1,1' & 2,2'	1.20/12H/t	12.08	1.16/12H/t	12.08
3,3' & 4,4'	3.57/8H/m	45.76	3.69/8H/m	45.76
5,5' & 6,6'	6.85/4H/d	113.66	7.47-7.56/8H/m	113.66
7,7' & 8,8'	7.28/4H/d	139.73	-	140.59
9	7.62/1H/s	127.42	7.03/1H/s	127.00
10	8.10/1H/d	128.90	7.92/1H/d	127.90
11	8.18/1H/d	129.02	8.28/1H/d	128.00
12		125.94		64.00
13		-		126.86
14		137.70		136.16
15		144.84		147.80
16		145.20		148.17
17, 18		154.91		154.98
19		170.03		176.61

Mechanism of impurity

The impurity was examined by hydrolysis of an intermediate to isosulfanblue. The probable mechanism of formation of impurity was shown in Figure 7.

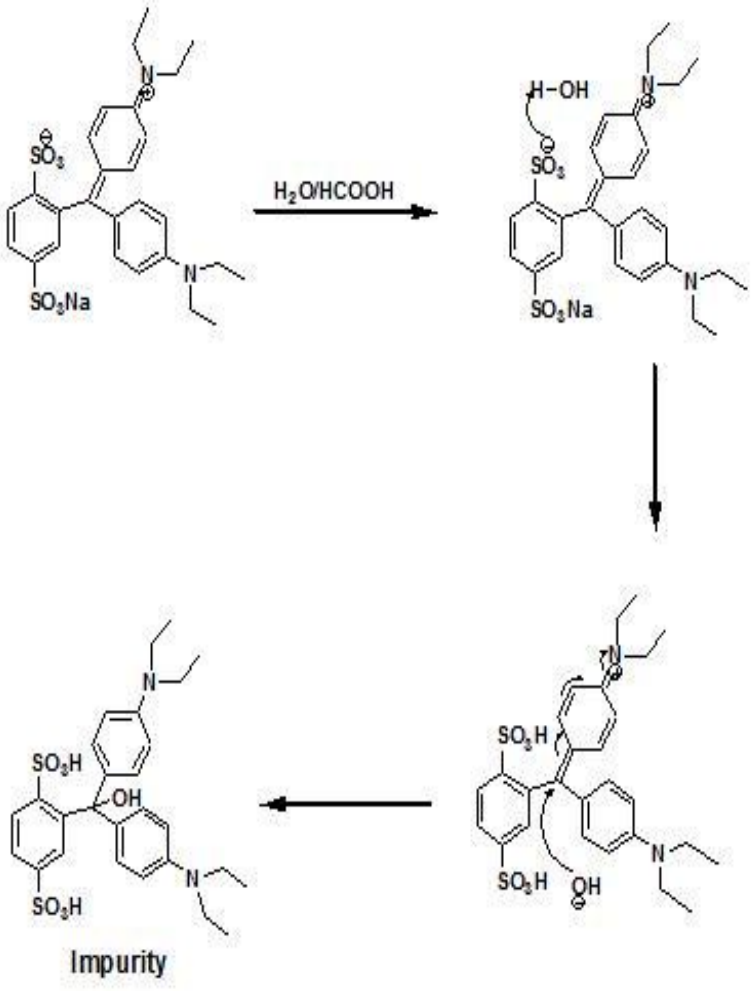


Figure 7: Mechanism for the formation of impurity 1

CONCLUSION

The process related impurity in isosulfanblue drug substance was identified, isolated and characterized by using HPLC (analytical and preparative), MASS, IR and NMR (^{1}H and ^{13}C-NMR) techniques.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the management of APL Research Centre (A Division of Aurobindo Pharma Ltd.), for allowing us to carry out the present work. The authors are also thankful to the colleagues of Chemical Research Department and Analytical Research Department for their Co-operation.

REFERENCES

[1] R. Kovi, S. Nampalli, P.X. Tharial, US 7,662,992B2, **2010**.
[2] L.L. Montgomery, A.C. Thorne, K.J. Van Zee, J. Fey, A.S. Heerdt, M. Gemignani, E. Port, J. Petrek, H.S Cody, P.I. Borgen, *Anesth. Analg*., **2002**, 95, 385-388.
[3] A.E. Giuliano, D.M. Kirgan, J.M. Guente, D.L. Morton, *Ann. Surg*., **1994**, 220(3), 391-401.
[4] P.J. Rubis, J. Tisnado, J.I. Hirsch, J. Capehart, *AJR*., **1982**, 139, 186-187.
[5] R. Ting, T. aguilera, J.L. Crisp, D.J. Hall, W.C. Eckelman, D.R. Vera, R.Y. Tsien, *Bioconjugate. Chem*., **2010**, 21, 1811-1819.
[6] D.S.B. Hoon, B. Taback, US 7,897,336B2, **2011**.
[7] Broderick-Villa, M.D. Gregory, M.D. Ko Albert, M.D.X. O'Connell Theodore, M.D. Guenther J Michael, M.D Danial Tare, M.D. DiFronzo L Andrew, *Cancer. J*., **2002**, 8(6), 445-450.
[8] B.K. Kulkarni, C. Sankar, M.K. Girish, H.T. Maurya, A.V. Arjun, S.S. Sandeep, K. Sumit US 7,534,911B2, **2009**.
[9] A. Islam, U.K. Ray, B. Sreenivasulu, M.S. Sundaram, 3509/CHE/2012A, **2014**.
[10] I. Bhatnagar, M.V. George, *Tetrahedron*., **1968**, 24, 1293-1298.
[11] S. Mashraqui, P. Keehn, *Synth. Commun*., **1982**, 12(8), 637-645.

[12] P.K. Hiranaka, L.M. Kleinman, E.A. Sokoloski, H.M. Fales, *Am. J. Hosp. Pharm*., **1975**, 32, 928-930.
[13] C. Chen, C. Lu, F. Mai, C. Weng, *J. Hazard. Mater*., **2006**, 137, 1600-1607.
[14] C. Chen, W. Zhao, J. Li, J. Zhao, *Environ. Sci. Technol*., **2002**, 36, 3604-3611.
[15] Guidance for industry, Q3B (R2) impurities in New Drug Product, International Conference on Harmonization, **2006.**