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Characterization and relative response factor determination of process related impurity in Naproxen by nuclear magnetic resonance spectroscopy

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ABSTRACT

In the impurity profile of naproxen around 0.6% unknown polar impurity was detected by high performance liquid chromatography (HPLC). The product ion spectrum of the impurity and Naproxen was recorded in LCMS/MS and the fragmentation pattern of the impurity was observed to be similar to the fragmentation pattern of Naproxen with only a difference of two atomic mass units. The high resolution mass spectrum (HRMS) of the impurity displayed a protonated molecular ion at *m/z* 229.0863, which corresponds to the pseudomolecular formula $C_{14}H_{13}O_3^+$. Based on LC/MS/MS, HRMS, 1D and 2D NMR data, the structure of the impurity was characterized as 2-(6-methoxynaphthalen-2-yl)acrylic acid. The acrylic acid impurity was synthesized in the laboratory and co injected in HPLC to confirm the retention time. RRF of the impurity was determined by ^{1}H NMR method and also by conventional HPLC slope method and the RRF values are found to be 6.11 and 5.64, respectively. The values are comparable and ^{1}H NMR method of RRF determination is complimentary and can be effectively used as an alternative method to conventional HPLC method especially in early stages of development when availability of impurity standards is not possible.

1. Introduction

Naproxen [(S)-2-(6-methoxynapthalen-2-yl)propanoic acid] is a non-steroidal anti-inflammatory drug used in reduction of pain, fever, inflammation and treatment of rheumatoid arthritis, degenerative joint disease, ankylosing spondylitis, acute gout and primary dismenorrea [1,2]. Only the (+) enantiomer of Naproxen possesses anti-inflammatory properties [3]. Naproxen is able to block cyclooxygenase enzymes (Cox-1 and Cox-2) which produce prostaglandins, a class of compounds which promote inflammation, pain and fever [4,5]. The most common side effects of Naproxen are gastrointestinal complaints, headache, light headedness and drowsiness [3]. Gastric and bleeding ulcers could be produced in some cases, and in a few cases renal failure, hepatic injury, urticaria, ecchymosis and vasculitis have been reported [3]. Several analytical methods have been reported in the literature for determination of Naproxen and related substances. In United State Pharmacopeia [6] and the British Pharmacopeia [7], thin layer chromatographic methods have been described for analysis of Naproxen related substances in formulations and raw materials. Liquid chromatography methods have been reported in the literature for individual and simultaneous determination of Naproxen and other anti-inflammatory drugs in biological fluids [8–11] and pharmaceutical preparations [12–14].

During HPLC analysis of Naproxen samples for related substances, an unknown polar impurity around 0.6% with respect to Naproxen was detected. For impurities in new drug substances, according to International Conference on Harmonisation (ICH) guidelines for a maximum daily dose ≤2 g/day of a drug substance, the reporting and identification thresholds are 0.05% and 0.10%, respectively [15]. Therefore it is a mandatory requirement from regulatory authorities to identify and characterize any unknown impurity present at or above 0.1% level in drug substance. Impurities in the drug substance can be accurately quantified only after correcting with relative response factors (RRF) of the respective impurities. In the conventional HPLC method, RRF values of impurities can be established by calculating the ratio of the responses of the impurities to the response of the drug substance at a specific wavelength. But in the early stages of process development, establishment of RRF values by HPLC is not possible due to the non-availability of impurity standards. Alternative methods have been described in the literature for establishing RRF using chemical luminescence detectors (CLND) [16], charged aerosol detectors (CAD) [17] and ^{1}H nuclear magnetic resonance spectroscopy (NMR) [18]. Both CLND and CAD detectors have significant limitations. CLND is only useful for nitrogen containing compounds and it is limited to

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a few mobile phases and volatile buffers [17]. CAD detectors are suitable only for compounds that can carry a charge under electrospray type ionization conditions and are compatible with only volatile buffers [18].

Webster et al. showed for the first time, the determination of relative response factors for chromatographic investigations using NMR spectrometry [18]. This method is not limited by the structure and mobile phase limitations as seen with CLDN and CAD methods. In the present study, the unknown polar impurity is characterized as 2-(6-methoxynaphthalen-2-yl)acrylic acid based on LC/MS/MS, HRMS, 1D- and 2D-NMR data and the details are discussed. No earlier reports have been discussed in the literature for this acrylic acid impurity and in the present study a recent approach using ^{1}H NMR along with conventional HPLC slope method for RRF determination of acrylic acid impurity has been described. RRF determination by this NMR method is very useful as an alternative to conventional HPLC based protocols especially in early stages of development when availability of impurity standards is not possible.

2. Materials and methods

2.1. Samples

The investigated samples of Naproxen were obtained from Process Research Department, Custom Pharmaceutical Services, Dr. Reddy's Laboratories Ltd., Hyderabad, India. The polar impurity was synthesized in the laboratory after identification by LC/MS. HPLC grade methanol and acetic acid were obtained from Merck, Mumbai, India. AR grade ammonium acetate salt was obtained from SD fine chemicals Ltd., Mumbai, India. Dimethylsulphoxide-*d*6 was purchased from Aldrich Chemical Co., USA. Water used for preparing mobile phase was purified using Millipore (MA 01821, USA) water purification system.

2.2. High-performance liquid chromatography (HPLC)

The analysis was performed on an Agilent 1100 series LC system, Agilent Technologies Inc., Santa Clara, CA, USA. Hypersil ODS column (4.6 mm × 100 mm, 5 μm) and mobile phase consisting of a mixture of sodium acetate trihydrate (pH 4.7; 0.04 M) – methanol (60:40, v/v), UV detection at 254 nm, flow rate of 1.5 mL/min was used for resolution of all the impurities. The column temperature was maintained at 40 °C. The Naproxen sample was prepared in methanol at 0.5 mg/mL concentration and 100 μL of sample solution was injected into HPLC system.

2.3. Mass spectrometry

An Agilent 1100 series LC system coupled to a triple quadrupole mass spectrometer (Agilent LC/MS/MS model 6410, Agilent Technologies Inc., Santa Clara, CA, USA) with electrospray ionization (ESI) source was used and ES ionization was done in positive mode. The ion source temperature was set at 300 °C and the ESI needle voltage was set at 3.0 kV. Nitrogen was used as the drying gas at a flow rate of 10 L/min and as the nebulizer gas at a pressure of 60 psi. For MS/MS studies, nitrogen was used as the collision gas with collision energy of 50 eV. Zorbax SB-C8 column (4.6 mm × 150 mm, 3.5 μm) with a mobile phase consisting of a mixture of ammonium acetate (pH 4.9; 0.01 M) – methanol (60:40, v/v) at a flow rate of 1.0 mL/min was used. The column temperature was maintained at 25 °C. The Naproxen sample was prepared in methanol at 1.0 mg/mL concentration and 100 μL of sample solution was injected in LC/MS/MS system.

The high resolution mass spectra were obtained from a Waters LCT Premier time-of-flight (TOF) mass spectrometer (Milford, USA) with ESI source. Resolution of the LC-TOF/MS was more than 5000.

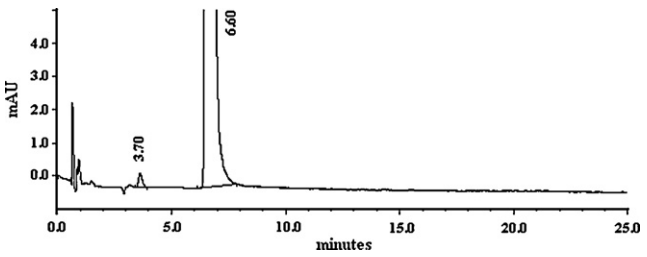

Fig. 1. HPLC chromatogram of Naproxen.

Leucine enkephalin ($C_{28}H_{37}N_5O_7$) was used as external lock-mass. The source block and desolvation temperatures were 90 °C and 180 °C, respectively. The nebulizer and desolvation gas (nitrogen) flows were 20 L/h and 450 L/h, respectively. The instrument parameters were used as capillary 3000 V, cone 25 V, extractor 2 V and MCP 2700 V. The acquisitions were done in scan mode. Data acquisition and processing were done using Mass Lynx V.4.0 software.

2.4. Preparation of solutions for RRF determination by HPLC

A solution of polar impurity and Naproxen was prepared by weighing 12.5 mg each into 25 mL volumetric flask and diluted up to the mark with methanol. From this solution, further dilutions were made to obtain concentrations 0.05% (w/w), 0.1% (w/w), 0.2% (w/w), 0.3% (w/w) and 0.5% (w/w). These solutions were injected in duplicate into HPLC system and the areas of impurity and Naproxen peaks were recorded at 254 nm.

2.5. Preparation of solutions for RRF determination by ^{1}H NMR

A solution of polar impurity and Naproxen was prepared by weighing 12.5 mg each in 5 mL of DMSO-d_6 solvent. The ^{1}H NMR for the solution was recorded in three replicates. Five different concentration solutions were made from the above solution and ^{1}H NMR spectra were recorded at each concentration. The solutions were further diluted and injected in duplicate into HPLC system and the areas of impurity and Naproxen peaks were recorded at 254 nm.

2.6. NMR spectroscopy

^{1}H NMR and two dimensional (2D) NMR experiments such as gradient Double Quantum Filtered Correlation Spectroscopy (gDQCOSY), gradient Heteronuclear Single Quantum Coherence Spectroscopy (gHSQC) and gradient Heteronuclear Multibond Coherence Spectroscopy (gHMBC) were performed on Varian Mercury plus 400 MHz NMR instrument at 25 °C in DMSO-*d6*. ^{13}C NMR experiments were performed on a Varian Gemini 200 MHz instrument, model 2000 at 25 °C in DMSO-*d6*. The ^{1}H and ^{13}C chemical shift values were reported on the δ scale in ppm, relative to TMS (δ = 0.00 ppm).

3. Results and discussion

3.1. Structural elucidation of polar impurity

An unknown polar impurity observed in Naproxen samples at 0.56 RRT in related substances HPLC method is shown in Fig. 1. A LC/MS compatible solvent system was developed as discussed in Section 2.3 and analysis was performed. The mass spectra of the impurity and Naproxen displayed protonated pseudomolecular ions at m/z = 229.00 and 231.00 with a difference of two m/z units. The product ion spectra of impurity and Naproxen are shown in Fig. 2. The product ion spectra showed daughter ions at m/z = 183.00

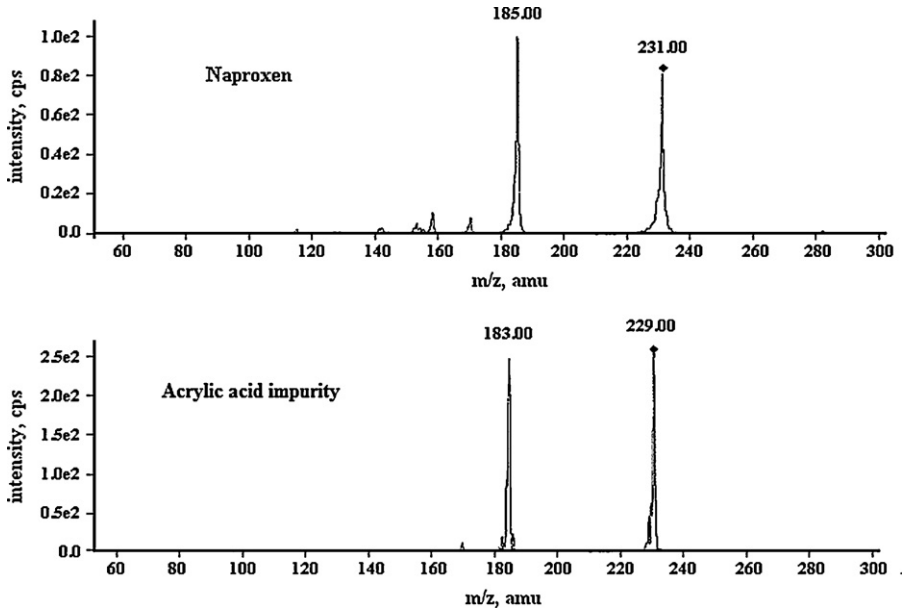

Fig. 2. Product ion spectra of Naproxen and acrylic acid impurity.

and 185.00, respectively, with a difference of two m/z units. The high resolution mass spectrum (HRMS) of the impurity displayed a protonated pseudomolecular ion at $m/z = 229.0863$, which corresponds to the pseudomolecular formula $C_{14}H_{13}O_3^+$. Based on the MS/MS data and elemental composition, the impurity structure was proposed and shown in Fig. 3. In product ion spectra, the daughter ions observed at $m/z = 183.00$ and 185.00 is due to sequential loss of a molecule of water and of a molecule of carbon monoxide from impurity and Naproxen and the corresponding fragmentation patterns are shown in Fig. 4.

The ^1H and ^{13}C NMR spectra of impurity and Naproxen are compared and significant changes are observed in the ^1H and ^{13}C signals at C5 and C4 positions. The chemical shifts of the C5 protons (structure shown in Fig. 3) of impurity were deshielded when compared

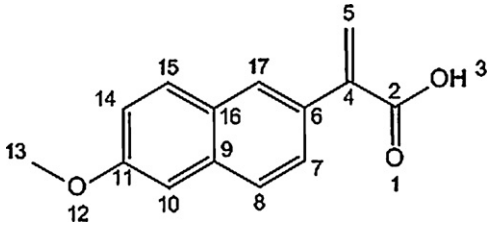

Fig. 3. Structural formula of the acrylic acid impurity.

to the chemical shift of the C5 protons (structure shown in Fig. 5) of Naproxen. The same trend was observed in ^{13}C chemical shifts at C4 and C5 positions. The C4 proton signal which is observed in the ^1H NMR spectrum of Naproxen (shown in Fig. 6) was not seen in the

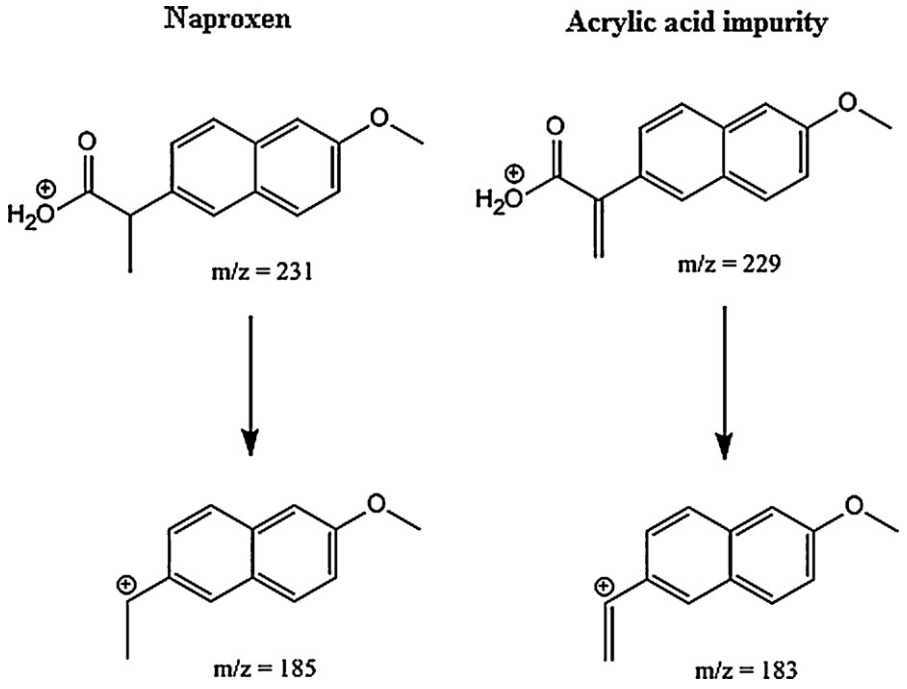

Fig. 4. MS/MS fragmentation path way for Naproxen and acrylic acid impurity.

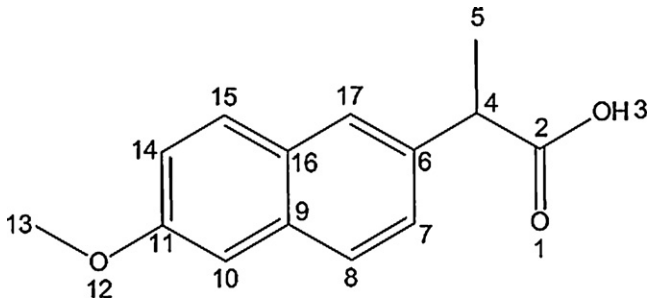

Fig. 5. Structural formula of Naproxen.

^{1}H NMR spectrum of impurity (shown in Fig. 7). The ^{13}C chemical shift of the C4 carbon of impurity was deshielded when compared to the chemical shift of the C4 carbon of Naproxen. No significant changes were observed in the chemical shifts of remaining ^{1}H and ^{13}C signals of impurity and Naproxen. ^{1}H NMR spectrum of the impurity displayed aromatic signals at $\delta = 7.53$ (1H, d, $J = 8.5$ Hz), $\delta = 7.78$ (1H, d, $J = 8.5$ Hz), $\delta = 7.32$ (1H, s), $\delta = 7.16$ (1H, d, $J = 9.0$ Hz), $\delta = 7.83$ (1H, d, $J = 8.5$ Hz) and $\delta = 7.90$ (1H, s). ^{13}C NMR spectrum displayed resonances at 168.05 (q), 141.53 (q), 131.84 (q), 133.84 (q), 157.62 (q), 128.00 (q), 126.26 (CH), 126.85 (CH), 105.69 (CH), 118.84 (CH), 129.72 (CH), 126.56 (CH), one methylene at 125.40 and one methyl at 55.18 ppm. The 2D NMR experiments were performed to

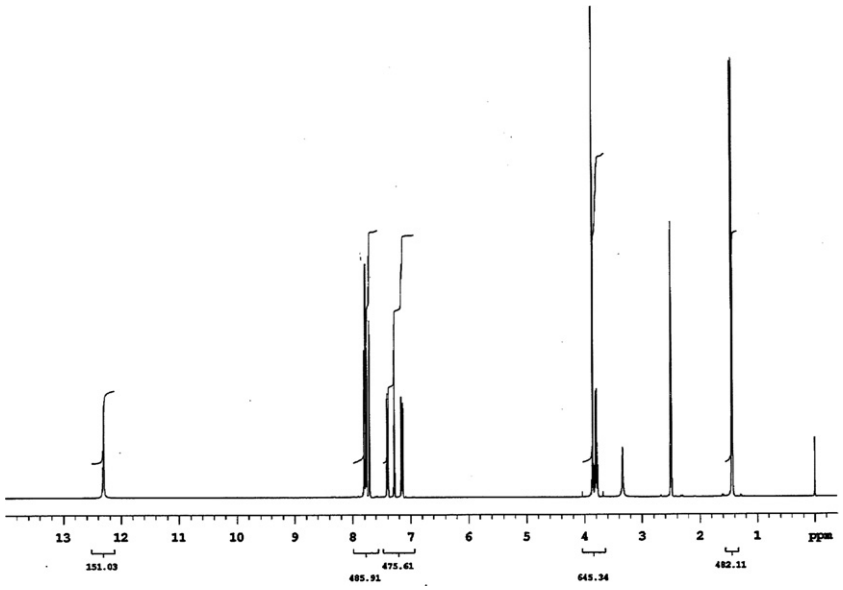

Fig. 6. Proton NMR spectrum of Naproxen.

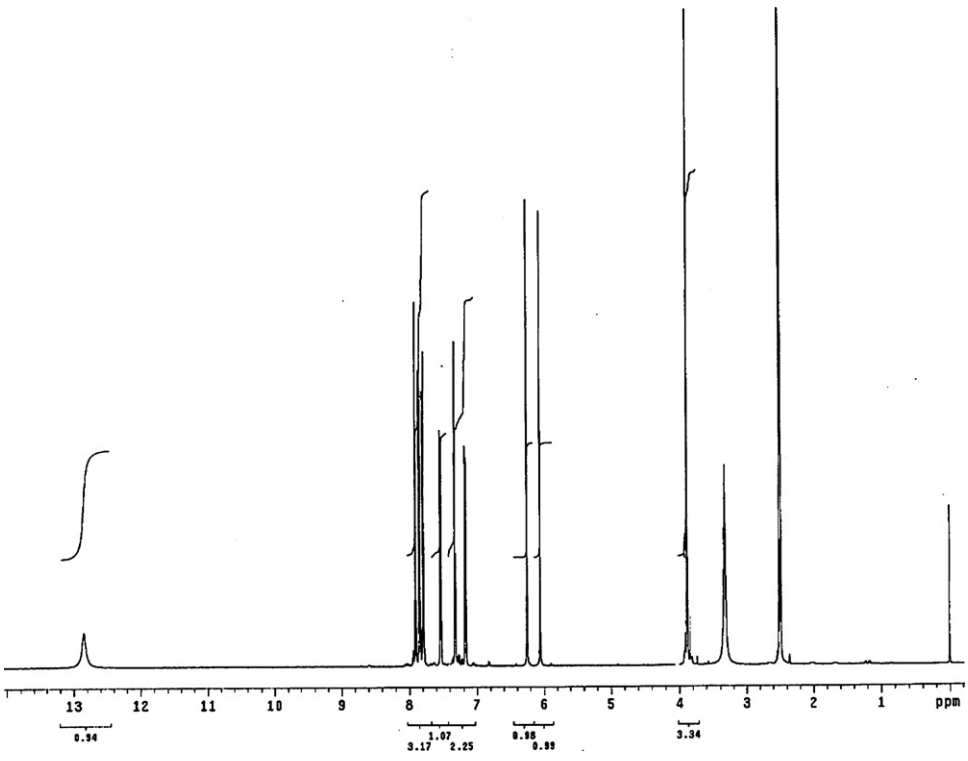

Fig. 7. Proton NMR spectrum of acrylic acid impurity.

Table 1
NMR assignments of impurity.

Position[a]	^{1}H	δ (ppm)	J (Hz)	gDQCOSY	^{13}C, δ (ppm)	gHSQC
1	–	–	–		–	–
2	–	–	–		168.05	–
3	1H	12.90	s (brs)		–	–
4	–	–	–		141.53	–
5	HaHb	6.05	s		125.40	5Ha, 6.05
		6.25	s			5Hb, 6.25
6	–	–	–		131.84	–
7	1H	7.53	d, 8.5	8H, 7.78	126.26	7H, 7.53
8	1H	7.78	d, 8.5	7H, 7.53	126.85	8H, 7.78
9	–	–	–		133.84	–
10	1H	7.32	s		105.69	10H, 7.32
11	–	–	–		157.62	–
12	–	–	–		–	–
13	3H	3.88	s		55.18	13H, 3.88
14	1H	7.16	d, 9.0	15H, 7.83	118.84	14H, 7.16
15	1H	7.83	d, 8.5	14H, 7.16	129.72	15H, 7.83
16	–	–	–		128.00	–
17	1H	7.90	s		126.56	17H, 7.90

s, singlet; d, doublet; brs, broad singlet; J, spin coupling constant;.
[a] Refers the structural formula in Fig. 3 for numbering.

identify the ^{1}H–^{1}H, ^{13}C–^{1}H couplings and the corresponding NMR assignments are shown in Table 1. The ^{1}H–^{1}H couplings observed in DQCOSY spectrum of impurity are shown in Fig. 8. Aromatic ^{1}H–^{1}H couplings are observed in DQCOSY spectrum, whereas the C5 and C13 proton couplings to other protons are not seen in the spectrum. The ^{13}C–^{1}H couplings observed in HSQC spectrum of the impurity are shown in Fig. 9. The HMBC spectrum of impurity displayed in Fig. 10 showed that the C5 protons are coupled (long range couplings) to aromatic C6 carbon and carbonyl carbon at C2 position and the corresponding HMBC linkages are shown in Fig. 11. The aromatic C6 carbon and C5 proton coupling confirms the attachment of side chain to C6 carbon. The DQCOSY, HSQC and HMBC spectra strongly support the structure proposed for impurity in Fig. 3.

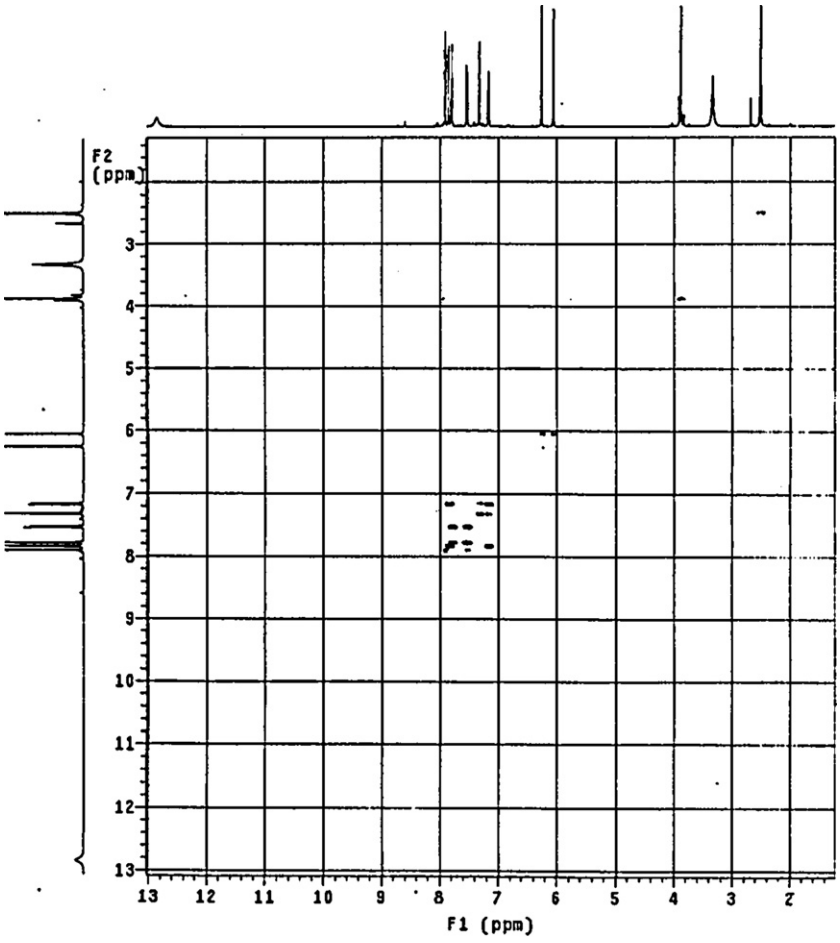

Fig. 8. DQCOSY NMR spectrum of acrylic acid impurity.

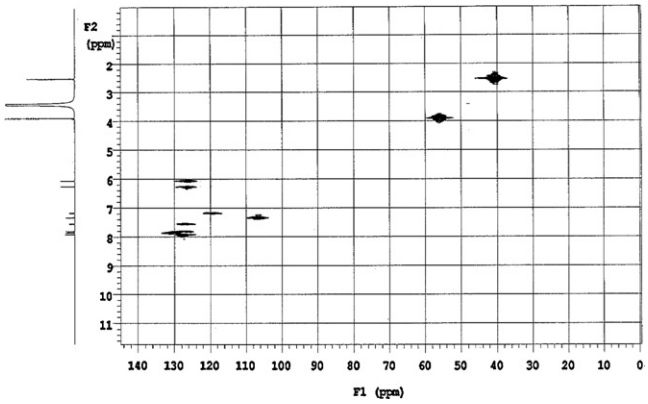

Fig. 9. HSQC NMR spectrum of acrylic acid impurity.

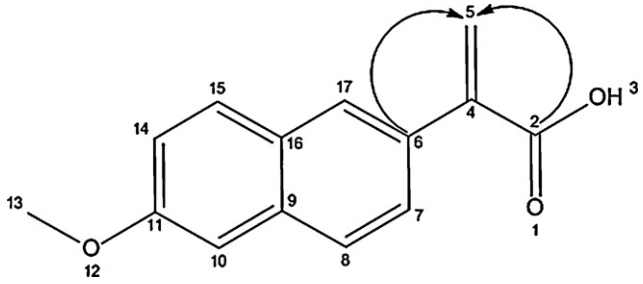

Fig. 11. HMBC linkages of C6 and C2 carbons to C5 protons.

3.2. Determination of relative response factor

3.2.1. RRF by HPLC

The relative response factor for the polar impurity was determined against Naproxen by conventional slope method using HPLC. The relative response factor was calculated using the below formula [17] and the results are shown in Table 2. The slope values for acrylic acid impurity and Naproxen are taken from linearity graphs as shown in Fig. 6.

$$\mathrm{RRF} = \frac{\mathrm{Slope}^1}{\mathrm{Slope}^2} \times \frac{\mathrm{Purity}^2}{\mathrm{Purity}^1} \quad (1)$$

where 1, 2 corresponds to impurity and Naproxen, respectively; Slope is obtained from the linearity curve.

The RRF value for the acrylic acid impurity obtained by HPLC using slope method is found to be 5.64.

3.2.2. RRF by ^{1}H NMR

Relative response factor for the polar impurity against Naproxen was also determined using ^{1}H NMR. The RRF calculation was done using the below formula [18]. The results are shown in Table 3.

$$\mathrm{RRF} = \frac{\mathrm{Area}^{\mathrm{UV}_1}}{\mathrm{Area}^{\mathrm{UV}_2}} \times \frac{I_2^{\mathrm{NMR}}}{I_1^{\mathrm{NMR}}} \times \frac{\mathrm{MW}_2}{\mathrm{MW}_1} \times \frac{N_1^{\mathrm{H}}}{N_2^{\mathrm{H}}} \quad (2)$$

where 1, 2 corresponds to acrylic acid impurity and Naproxen, respectively; $\mathrm{Area}^{\mathrm{UV}}$, HPLC peak area; I^{NMR}, Integral value obtained form ^{1}H NMR spectrum; MW, molecular weight; and N^{H}, number of hydrogen atoms in the integration.

The RRF value for the polar impurity from ^{1}H NMR is found to be 6.11. The RRF values obtained from ^{1}H NMR method and HPLC method are comparable.

4. Ames test

Ames test is a biological assay, which was performed to assess the mutagenic potential of acrylic acid impurity. Ames test results showed that the acrylic acid impurity did not induce mutation in the *Salmonella typhimurium* bacterial strains, when treated up to the precipitating concentrations (5000 μg per plate) both in the presence and absence of rat liver metabolic activation system by both plate incorporation and pre-incubation methods.

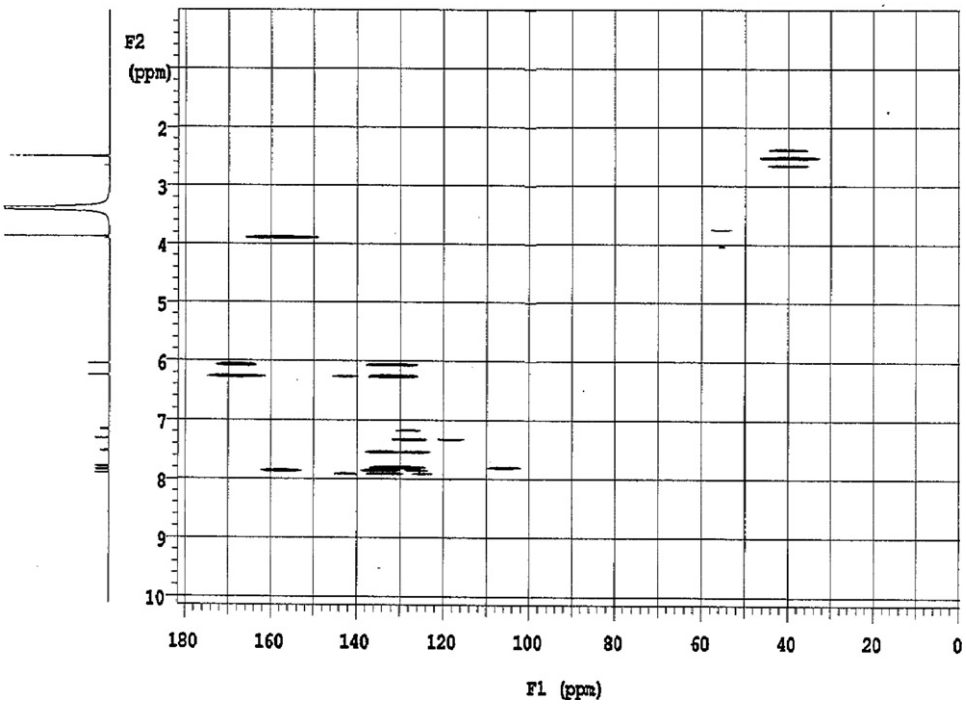

Fig. 10. HMBC NMR spectrum of acrylic acid impurity.

Table 2
RRF determination by HPLC.

% Level	Acrylic acid impurity		Naproxen	
	Concentration (μg/mL)	Avg. area of peak (mV/s)	Concentration (μg/mL)	Avg. area of peak (mV/s)
0.05	0.0238	10.082	0.0251	2.444
	0.0238	10.217	0.0251	2.692
0.1	0.0476	20.296	0.0501	4.101
	0.0476	20.243	0.0501	4.085
0.2	0.0952	39.943	0.1003	8.096
	0.0952	39.953	0.1003	7.700
0.3	0.1428	60.067	0.1504	11.619
	0.1428	60.044	0.1504	11.541
0.5	0.2379	98.553	0.2507	18.965
	0.2379	98.523	0.2507	18.932
	Correlation coefficient (R)	0.9999	Correlation coefficient (R)	0.9997

Table 3
RRF determination by ^{1}H NMR.

Concentration (μg/mL)	Acrylic acid impurity		Naproxen		RRF
	Avg. area of peak (mV/s)	Integral by ^{1}H NMR	Avg. area of peak (mV/s)	Integral by ^{1}H NMR	
0.7211	279011	945.70	49498	1000.00	6.07
1.4423	510904	930.28	90207	1000.00	6.20
1.9471	697181	944.73	123291	1000.00	6.10
2.8846	1120118	944.77	197319.5	1000.00	6.12
3.6057	1483097	951.94	261198.5	1000.00	6.07
				Average RRF	6.11

5. Conclusions

The unknown polar impurity observed in the range of 0.6% at 0.56 RRT by HPLC was identified by LC/MS. Based on the spectral data (MS and NMR), the structure of the impurity was characterized as 2-(6-methoxynaphthalen-2-yl)acrylic acid. RRF of the impurity was determined by conventional HPLC slope method, ^{1}H NMR method and the values are found to be 5.64 and 6.11, respectively. The RRF values are comparable and ^{1}H NMR method provides useful supporting information. The ^{1}H NMR can be used as an alternative method to conventional HPLC method especially when availability of impurity standards is not possible during early stages of development.

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