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Study of impurity carryover and impurity profile in Febuxostat drug substance by LC–MS/MS technique

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ABSTRACT

Febuxostat is used in the treatment of hyperuricemia and gout. Several impurities were detected in Febuxostat drug substance. Impurities were identified with the help of LC–MS/MS and were characterized after synthesis by IR and NMR. Reverse phase gradient system was used with Kromasil C18, 150 mm × 4.6 mm, 5 μm particle size column for the separation of impurities. Q-TOF mass spectrometer with electrospray ionization (ESI) source was used and operated in ESI positive mode, which gives exact mass up to four decimal places and fragmentation with mass accuracy, it is useful for the identification of impurities. Four impurities were identified as amide, sec-butyl, des-cyano and des-acid in Febuxostat drug analog. These impurities were further confirmed by NMR and FT-IR spectral data.

1. Introduction

Febuxostat (2-(3-cyano-4-isobutoxyphenyl)-5-methyl-1,3-thiazole-4-carboxylic acid) is an orally administered nonpurine selective inhibitor of xanthine oxidase (XO) that is indicated for use in the treatment of hyperuricemia and gout [1]. The enzyme that catalyzes the synthesis of uric acid from hypoxanthine and xanthine. In vitro studies have shown that Febuxostat is a potent ligand and inhibitor of both the oxidized and reduced forms of XO. Treatment with Febuxostat resulted in a significant reduction of sUA (serum urate) levels at all dosage. Febuxostat therapy is safe and well tolerated [2–5]. Regulatory agencies have heightened their scrutiny of the safety profile. As a result increased emphasis has been placed on the identification, formation, fate and process control of impurities in starting materials, raw materials, isolated intermediates and the active pharmaceutical ingredients (API). Impurity profiling (i.e., the identity as well as the quantity of impurity in the pharmaceuticals), is now gaining critical attention from regulatory authorities [6,7].

Febuxostat is not yet official in any of the pharmacopeia. Since impurities in pharmaceuticals can cause undesirable side effects in patients, guidelines on impurities in new drug substance and the identification and quantification of impurities have been issued by International conference on harmonisation [8,9].

Febuxostat received marketing approval by the European Medicines Agency on April 21, 2008 and was approved by the U.S. Food and Drug Administration on February 16, 2009.

The present studies have been conducted for the impurity profile of Febuxostat API and carryover impurity from the intermediate stage and raw materials using LC–MS Q-TOF instrument. The Q-TOF instrument has been used for the impurity profile which gives automated exact mass measurement. The high quality data delivered by the Q-TOF can provide information on elemental composition and structural characteristics providing excellent specificity for identifying compounds in complex matrices. The structure of impurities has been further confirmed by IR, NMR and mass after isolation.

2. Experimental

2.1. Materials

Febuxostat and key starting materials used in this study were synthesized by Unimark Remedies Ltd. (Ahmedabad, India). Ammonium acetate, acetonitrile and acetic acid were purchased from Merck Specialities Private Limited (India). Purified water was used using Millipore Milli-Q Gradient A10 system (Milford, MA, USA) purification system.

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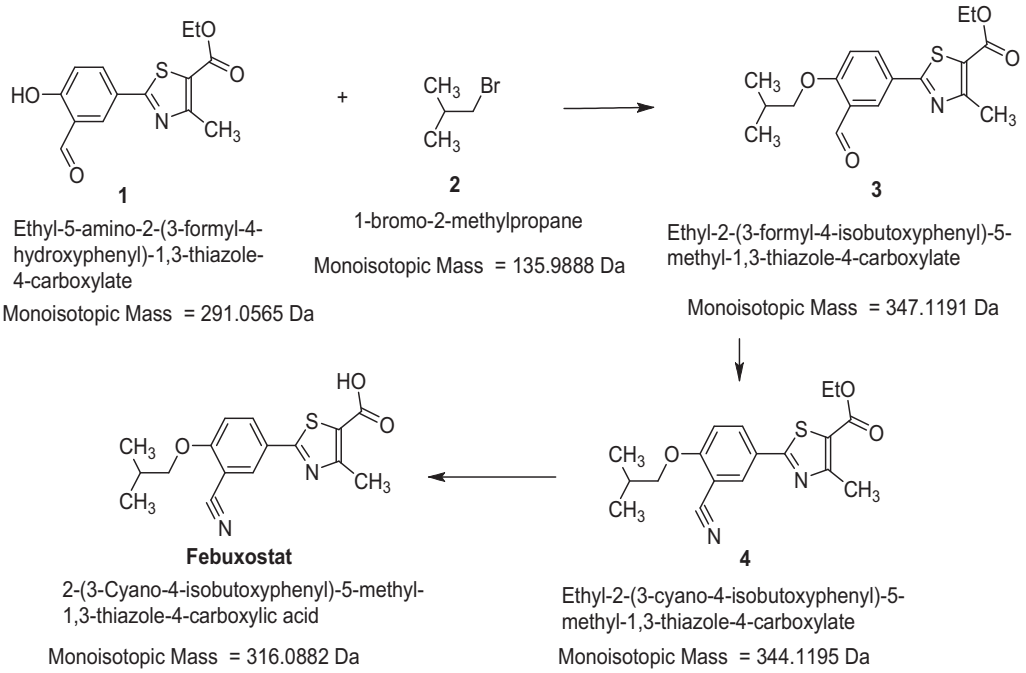


Fig. 1. Synthesis of Febuxostat.

2.2. Instrumentation

An LC–MS system equipped with the HPLC system of Waters Alliance (Waters, Milford, MA, USA) consisting of a 2695 quaternary pump and auto sampler, and a 2996 photodiode array detector. A hybrid quadrupole-time-of-flight mass spectrometer Q-TOF micro (Waters, Milford, MA, USA) equipped with electrospray ionization (ESI) source and operated in positive ESI mode. MassLynx V4.1 software (Waters) was used. Nitrogen was used for cone gas flow and desolvation gas flow with flow rates of 50 l/h and 500 l/h respectively. The source and desolvation temperatures were 120 and 230 °C, respectively. The capillary voltage was 3500 V and the sample cone voltage was 15 V. The collision energy was set to 6 V. Mass spectra were acquired over an m/z range of 100–1000 with a resolution of approximately 5000 at full-width half-maximum. For the MS/MS operation, argon was used as a collision gas. Accurate masses were measured by comparison to a reference compound, leucine enkephalin (m/z of the protonated molecule 556.2771) infused into the lock spray reference channel.

2.3. Analytical methods

HPLC method used for Febuxostat API: the buffer solution used for the preparation of Mobile phase A consists of 0.01 M aqueous ammonium acetate and its pH was adjusted to 3.5 with Trifluoro acetic acid. Acetonitrile was used as Mobile phase B.

Kromasil C18, 150 mm × 4.6 mm, 5 μm particle size column was used with a time gradient program of T (min)/% of Mobile phase B (v/v). Initial gradient of Mobile phase B starts with 32% and at 15 min it was 48%, and changed to 54% at 32 min and reached 85% at 40 min. The ratio being continued up to 50 min and at 53 min it was brought back to initial composition (32%), which was continued up to 60 min with a flow rate of 1.0 ml/min and column eluent, was monitored by UV detector at 315 nm. Column oven temperature was 30 °C. The injection volume was 10 μl. Diluent was the mixture of water and acetonitrile in the ratio of (20:80) and sample concentration was 1 mg/ml.

3. Result and discussion

The synthetic route of Febuxostat is shown in Fig. 1. Analysis of Febuxostat active pharmaceutical ingredients (API) by HPLC indicated the presence of new impurities. Further these impurities were not getting easily purged out from the Febuxostat API. The study of LC–MS/MS of starting materials intermediates and API indicates that impurities were carried over from the starting materials, intermediates and generated in the process. This was identified with the use of accurate mass measurement and fragment patterns. The HPLC chromatograms of Febuxostat and intermediates are shown in Fig. 2, the retention rime (RT) and relative retention time (RRT) of impurities are given in Table 1 with LC–MS/MS information.

3.1. Impurity-1

The mass (M+H) of Febuxostat observed in LC–MS was 317.0962 Da, from the structure of Febuxostat the index of hydrogen deficiency [10] of protonated Febuxostat is 9.5 due to two rings, six double bonds and one triple bond (2 + 6 + 2 − 0.5) (0.5 due to protonated molecule) and the LC–MS analysis is matching with this value.

Impurity-1 eluted at 0.46 RRT (Fig. 2a) which was identified with the help of LC–MS/MS analysis. Mass (M+H) of the compound was found 335.1046 Da. In MS/MS impurity lost NH_2 group, then $CONH_2$ and then isobutyl group. Index of hydrogen deficiency of the impurity was found to be 8.5, which is one less than Febuxostat and molecular weight of impurity found 18 Da higher than Febuxostat which means change in only either double bond or triple bond. From the LC–MS/MS analysis, the proposed molecule is 2-(3-carbamoyl-4-isobutoxyphenyl)-4-methyl-1, 3-thiazole-5-carboxylic acid and the proposed molecule has two rings and seven double bonds i.e. 2 + 7 − 0.5 (0.5 due to protonated molecule) = 8.5 Index of hydrogen deficiency, which is matching with the value found. Impurity-1 could have originated from the synthesis of 3 to **4** (Fig. 3). In **4** (intermediate stage) impurity eluted at RRT 0.37 (Fig. 2b) with m/z 363.1399 Da (M+H). Molecular weight and

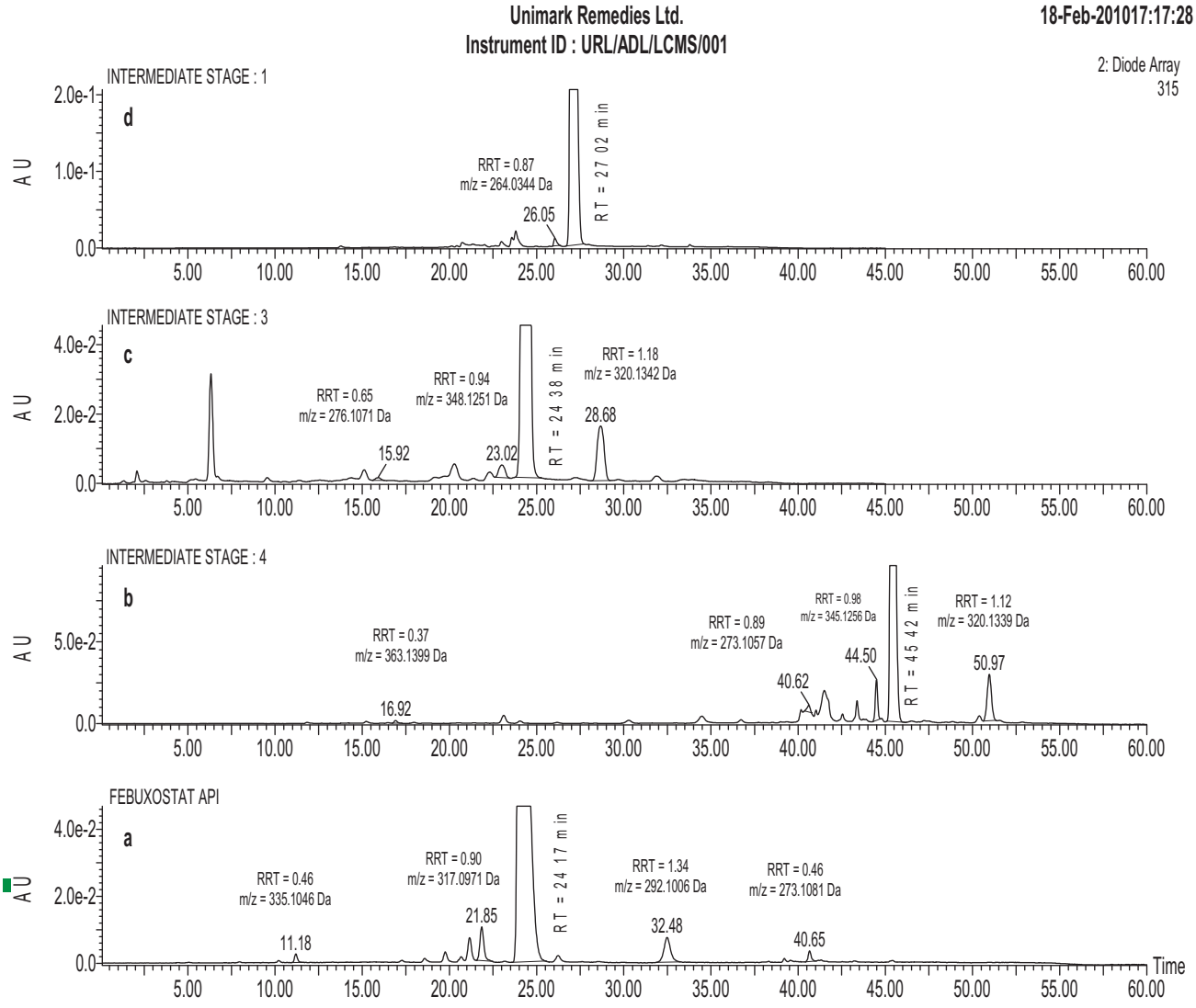


Fig. 2. (a) Chromatograph of Febuxostat API; (b) chromatograph of intermediate 4; (c) chromatograph of intermediate 3; (d) chromatograph of intermediate 1.

fragment pattern of corresponding impurity in **4** matched with ethyl 2-(3-carbamoyl-4-isobutoxyphenyl)-4-methyl-1,3-thiazole-5-carboxylate and upon further reaction it would convert into impurity-1 in Febuxostat. Impurity-1 was finally identified as 2-(3-carbamoyl-4-isobutoxyphenyl)-4-methyl-1, 3-thiazole-5-carboxylic acid, Fig. 3 represents the formation of impurity-1 and fragmentation pattern in MS/MS. The impurity was synthesized and confirmed with the help of IR and NMR and named as *amide impurity*.

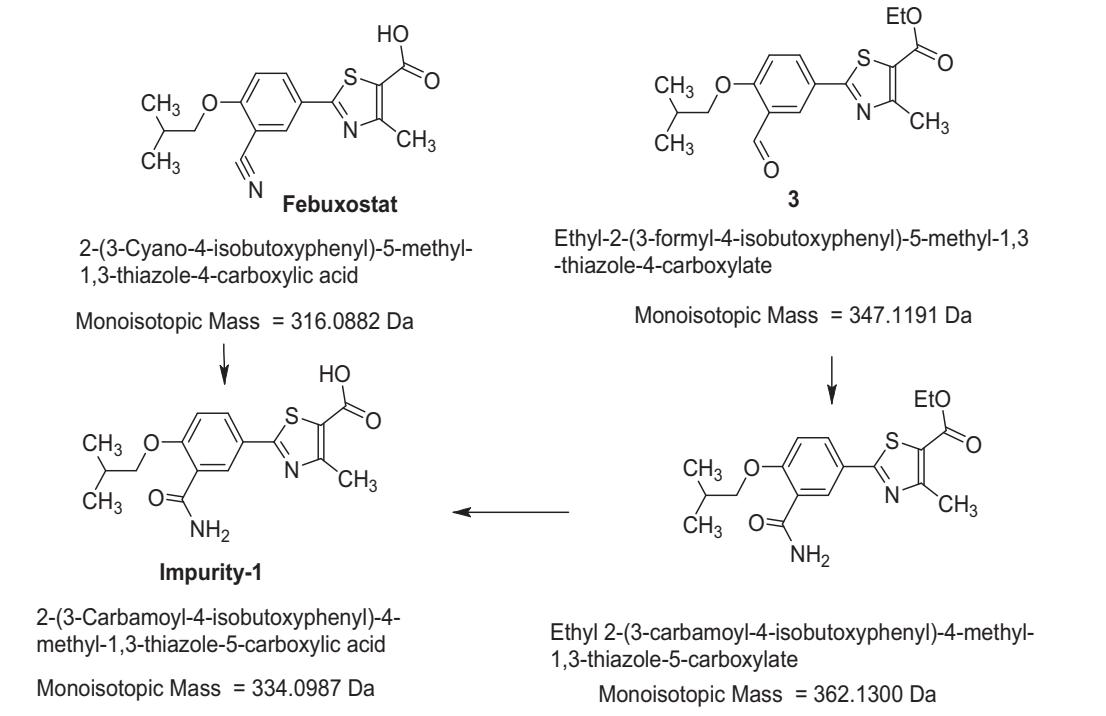
3.2. Impurity-2

Impurity-2 eluted at 0.90 RRT (Fig. 2a) which was identified with the help of LC–MS/MS analysis. Mass (M+H) of the compound

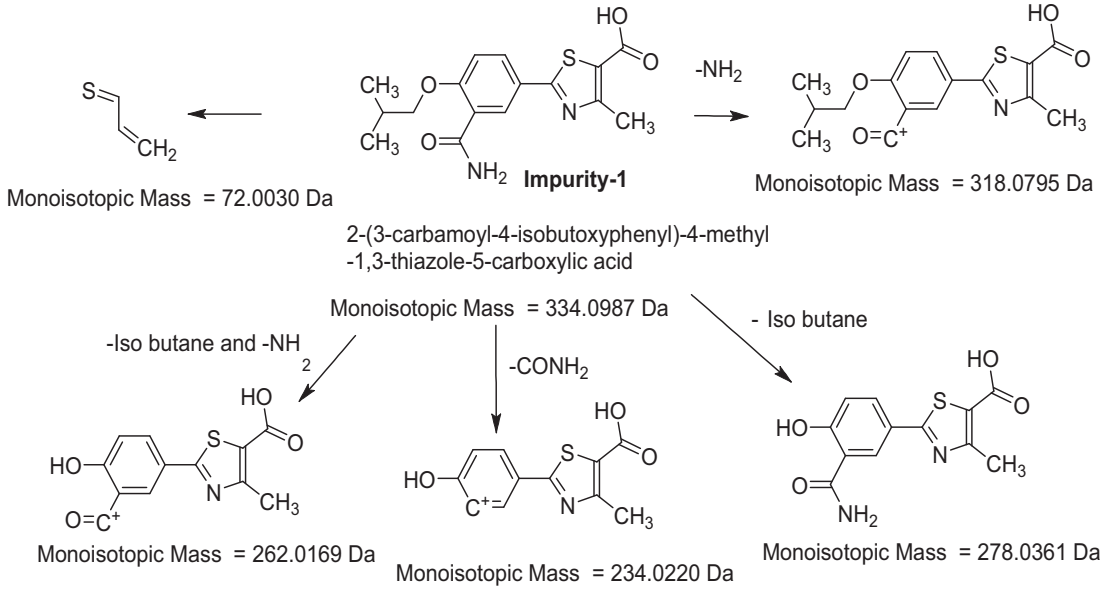
Table 1

Sr. no.	Impurity name	Exact molecular weight	Observed molecular weight (M+H)	[a]ppm error	[b]i-FIT[a] (isotope matching)	Index of hydrogen deficiency	MS–MS pattern	Probable formula (from the software prediction)
1	Febuxostat	316.0882	317.0962	0.6	4.0	9.5	–	$C_{16}H_{16}N_2O_3S$
2	Impurity-1 RT = 11.18 min RRT= 0.46	334.0987	335.1046	−6	3.7	8.5	318.1, 280.0, 279.0, 262.0, 234.0,	$C_{16}H_{19}N_2O_4S$
3	Impurity-2 RT = 21.85 min RRT = 0.90	316.0882	317.0971	3.5	0.2	9.5	261.0, 217.1, 145.0, 73.0	$C_{16}H_{17}N_2O_3S$
4	Impurity-3 RT = 32.48 min RRT = 1.34	291.0929	292.1006	−0.3	4.1	7.5	236.1, 192.1, 120.0, 73.0	$C_{15}H_{18}NO_3S$
5	Impurity-4 RT = 40.65 min RRT = 1.68	272.0983	273.1081	7.0	7.3	8.5	217.0, 145.0, 73.0	$C_{15}H_{17}N_2OS$

[a] ppm error is the difference between the calculated mass and the entered mass in parts per million.
[b] The i-FIT is a measure of likelihood that the isotopic pattern of the elemental composition matches a cluster of peaks in the spectrum. The lower the i-FIT value is, the better the fit.



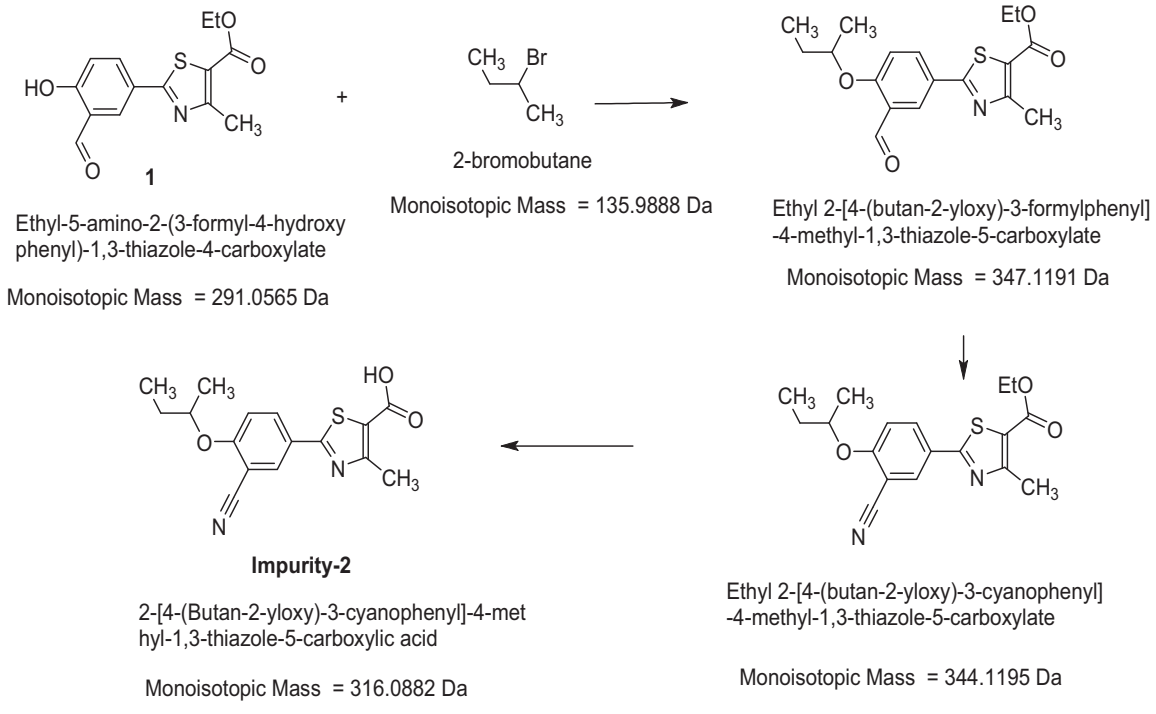
(a) Impurity-1 formation during synthesis



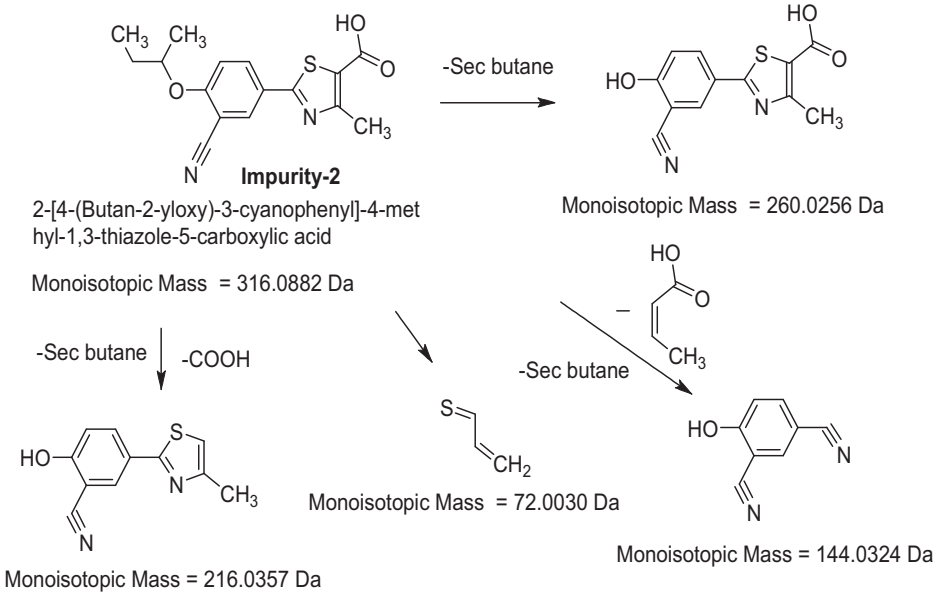
(b) Fragmentation of impurity-1 in MS-MS

Fig. 3. (a) Formation of impurity-1 during synthesis of Febuxostat; (b) fragment pattern of impurity-1 in MS/MS.

was found 317.0971. In MS/MS key fragmentations of compound are 261.0, 217.1, 145.0 and 73.0 Da. Index of hydrogen deficiency of the impurity was found to be 9.5, which is same as Febuxostat and mass of the impurity is also same as Febuxostat. From the LC–MS/MS analysis, the proposed molecule is 2-[4-(butan-2-yloxy)-3-cyanophenyl]-4-methyl-1,3-thiazole-5-carboxylic acid and proposed molecule has two rings, six double bonds and one triple bond i.e. 2 + 6 + 2 – 0.5 (0.5 due to protonated molecule) = 9.5. Index of hydrogen deficiency, which is matching with the value found. Impurity-2 could have formed due to presence of 2-bromobutane in **2** (Fig. 4), which would react with **1** and corresponding impurity was eluted in **3** (intermediate stage) at 0.94 RRT (Fig. 2c) with *m*/*z* 348.1251 Da (M+H), which would further react and form corresponding impurity in **4** (intermediate stage), eluted at 0.98 RRT (Fig. 2b) with *m*/*z* 345.1256 Da (M+H). Again it reacted and was carried over in Febuxostat as an impurity-2, exact mass and fragmentation pattern of impurity in each stage matches with respective proposed impurities. The impurity-2 was identified as 2-[4-(butan-2-yloxy)-3-cyanophenyl]-4-methyl-1,3-thiazole-5-carboxylic acid. Fig. 4 represents the formation of impurity-2 and its fragmentation pattern in MS–MS. The impurity was synthesized and finally



(a) Impurity-2 formation during synthesis



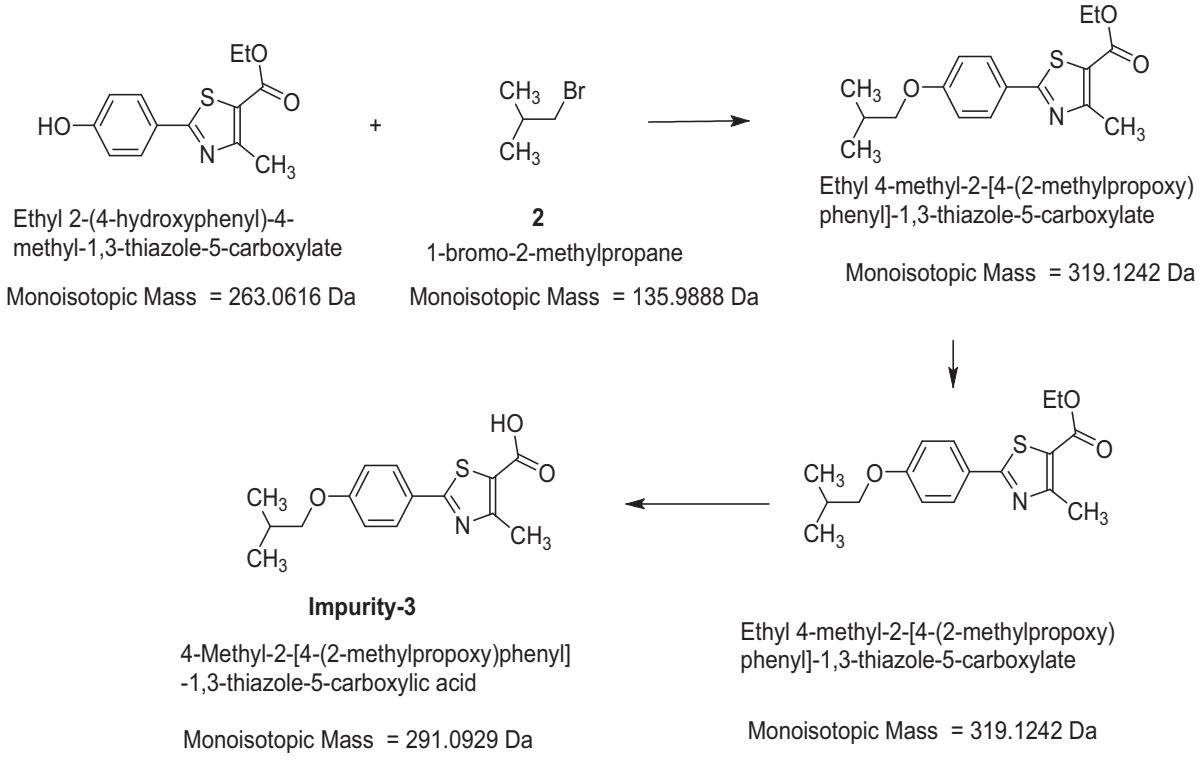
(b) Fragmentation of impurity-2 in MS-MS

Fig. 4. (a) Formation of impurity-2 during synthesis of Febuxostat; (b) fragment pattern of impurity-2 in MS/MS.

confirmed with the help of IR and NMR, and named as *sec-butyl impurity*.

3.3. Impurity-3

Impurity-3 eluted at 1.34 RRT (Fig. 2a) which was identified with the help of LC–MS/MS analysis. Mass (M+H) of the compound was found 292.1006 Da. In MS/MS data key fragments of the compound are 236.1, 192.1, 120.0 Da. Index of hydrogen deficiency of the impurity was found 7.5, it has two rings and six double bonds i.e. 2 + 6 – 0.5 (0.5 due to protonated molecule), which is two less than Febuxostat and molecular weight was found 25 Da less than Febuxostat. Molecular weight of impurity is odd, while molecular weight of Febuxostat is even. As per nitrogen rule, the impurity has a more or less nitrogen than Febuxostat. From the LC–MS/MS analysis the proposed molecule is 4-methyl-2-[4-(2-methylpropoxy) phenyl]-1,3-thiazole-5-carboxylic acid and the proposed molecule has two rings and six double bonds i.e. 2 + 6 – 0.5 (0.5 due to protonated molecule) = 7.5. Index of hydrogen deficiency, which is matching with value found. Formation of impurity-3 could have



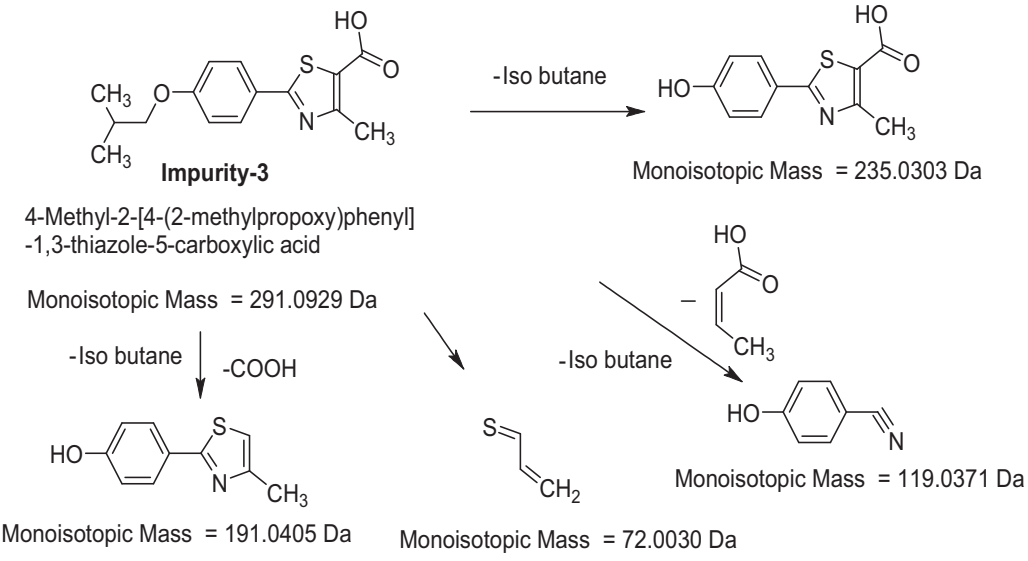


Fig. 5. (a) Formation of impurity-3 during synthesis of Febuxostat; (b) fragment pattern of impurity-3 in MS/MS.

been due to presence of ethyl 2-(4-hydroxyphenyl)-4- methyl-1,3-thiazole-5-carboxylate in **1** (Fig. 5), which was eluted at 0.87 RRT (Fig. 2d) with molecular weight 264.0344 Da (M+H). It would react with **2** and carried over in **3** with 1.18 RRT (Fig. 2c) with molecular weight 320.1342 Da (M+H), which would react and forward in **4** and eluted at 1.12 RRT (Fig. 2b) with molecular weigh 320.1339 Da (M+H) and again carried over in Febuxostat as impurity-3. Exact mass and fragmentation pattern of impurity in each step have matched with respective proposed impurity. Impurity was finally identified as 4-methyl-2-[4-(2-methylpropoxy) phenyl]-1,3-thiazole-5-carboxylic acid. Fig. 5 represents the formation of impurity-3 and the fragmentation pattern in MS/MS. Finally impurity-3 was synthesized and confirmed as *des-cyano impurity* with the help of IR and NMR.

3.4. Impurity-4

Impurity-4 eluted at 1.68 RRT (Fig. 2a) which was identified with the help of LC–MS/MS analysis. Mass (M+H) of the compound was found as 273.1081 Da. According to the MS/MS data impurity

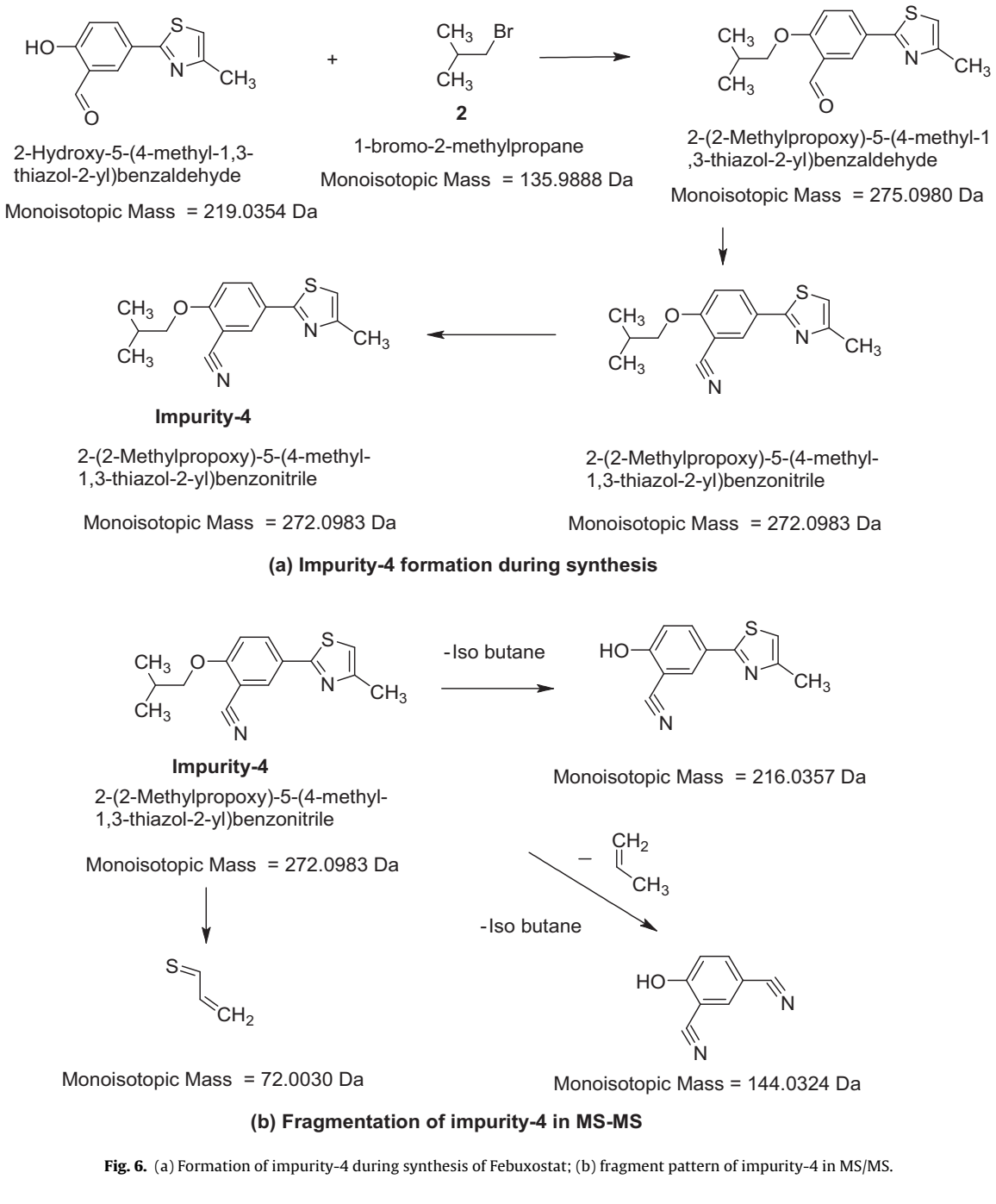


Fig. 6. (a) Formation of impurity-4 during synthesis of Febuxostat; (b) fragment pattern of impurity-4 in MS/MS.

had lost an isobutyl group and there was no acid fragmentation. Index of hydrogen deficiency of the impurity was found to be 8.5, which is one less than Febuxostat and molecular weight of impurity found was 44 Da less than Febuxostat which means loss of either a double bond or a triple bond. It is not due to loss of ring structure as that would have reduced the index of hydrogen deficiency by more than one unit. From the LC–MS/MS analysis the proposed molecule is 2-(2-methylpropoxy)-5-(4-methyl-1,3-thiazol-2-yl) benzonitrile and the proposed molecule has two rings, five double bonds and one triple bond i.e. 2 + 5 + 2 – 0.5 (due to protonated molecule) = 7.5. Index of hydrogen deficiency, which is matching with the value found. Formation of impurity-4 is due to presence of 2-hydroxy-5-(4-methyl-1,3-thiazol-2-yl) benzaldehyde in **1** (Fig. 6), It would react with **2** and carried over in **3**. The corresponding impurity was eluted in **3** at 0.65 RRT (Fig. 2c) with molecular weight would be 276.1071 Da (M+H), it would further react and be carried over in **4** and eluted at 0.89 RRT (Fig. 2b) with molecular weight 273.1057 Da (M+H) which is carried over to Febuxostat as impurity-4. Exact mass and fragmentation pattern of impurity in each step has matched with the respective proposed impurity. Finally impurity was identified as 2-(2-methylpropoxy)-5-(4-methyl-1,3-thiazol-2-yl) benzonitrile. Fig. 6 represents the formation of impurity-4 and fragment pattern in MS–MS. This was confirmed as *des-acid impurity*.

MS/MS fragment patterns of impurities is given in Fig. 7. NMR and IR spectroscopy data are given in Table 2.

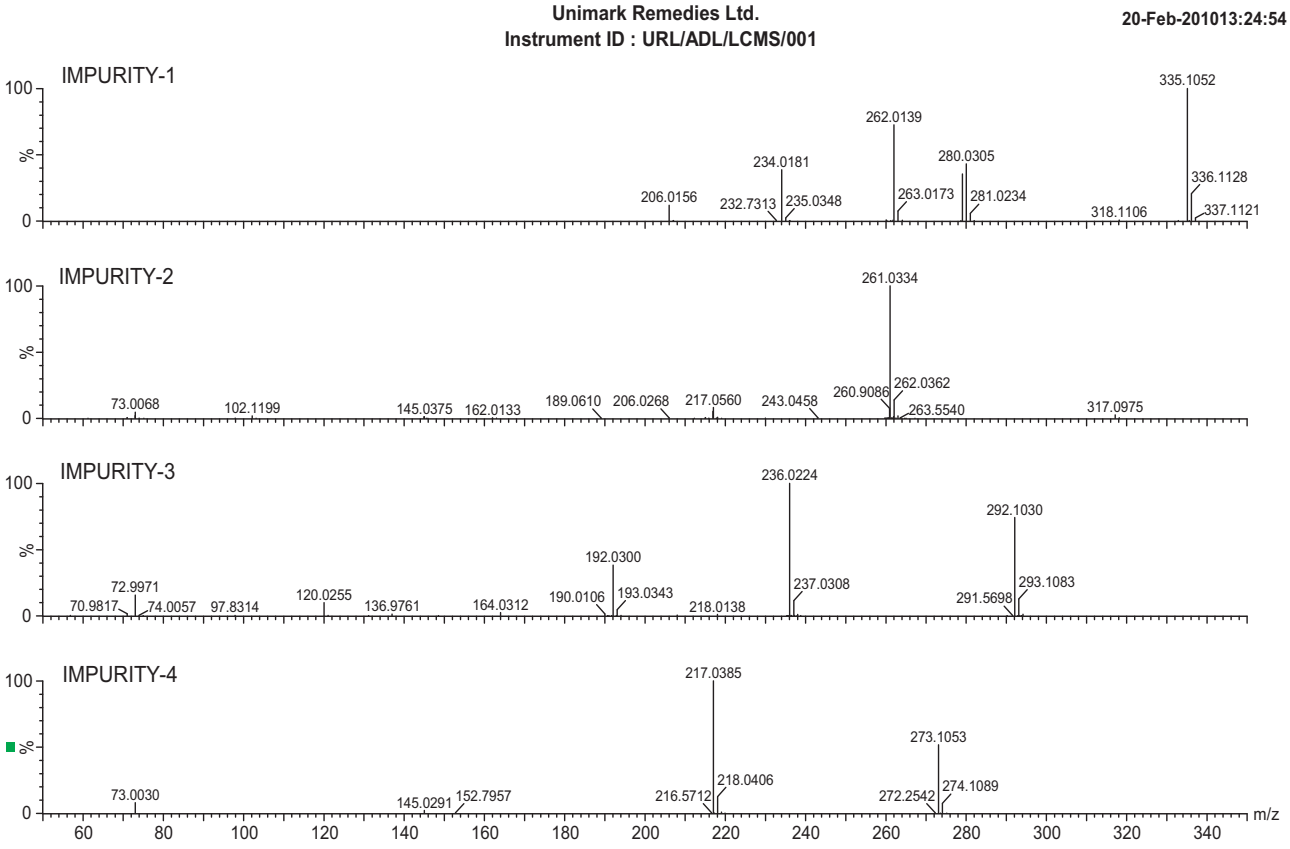
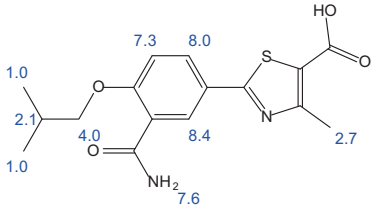
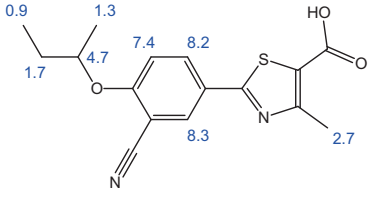
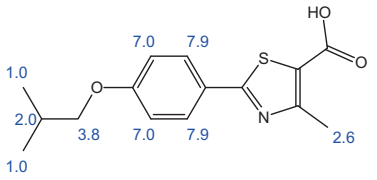


Fig. 7. MS/MS fragmentation pattern of impurities.

Table 2
Characterization data of impurity.

Sr. no.	Name of impurity	Test performed		
		Mass spectrometry	Infrared absorption	Proton nuclear magnetic resonance spectrometry
1	Impurity-1	335.1070	1254.79 –C–O– stretching 1435.42 –CH_3–C– of thiazole 1600.32 –CH═CH– stretching 1649.09 C═O stretching of acid 1675.07 C═O stretching of amide N–H of amide	
2	Impurity-2	317.0957	1281.12 –C–O– stretching 1426.78 –CH_3–C– of thiazole 1603.12 –CH═CH– stretching C═O stretching of acid 2226.98 C≡N stretching	
3	Impurity-3	292.1030	1253.38 –C–O– stretching 1437.36 –CH_3–C– of thiazole 1605.13 –CH═CH– stretching C═O stretching of acid	
4	Impurity-4[a]	273.1108	Not characterized	Not characterized

[a] Characterization data of impurity-4 is not available due to non availability of purified impurity.

4. Conclusion

With the help of the above study, impurities present in Febuxostat drug substance was identified by using Q-TOF mass spectrometer. Q-TOF technique facilitated structural identification. This technique was effective for the rapid identification of impurities. Result shows impurities present in Febuxostat drug substance are found from route synthesis and also from the impurity of raw materials as carryover impurities. The data presented is helpful for the understanding impurity profile of Febuxostat drug substance and also can be helpful to control the impurities in final product. This study can be helpful in identification and back integration of impurities present in active pharmaceutical ingredients and drug products.

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