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Research Article

ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF GENOTOXIC IMPURITIES IN FEBUXOSTAT DRUG SUBSTANCE AND PRODUCTS

BALAJI N*, SAYEEDA SULTANA

Department of Chemistry, St. Peter's University, Avadi, Chennai - 600 054, Tamil Nadu, India. Email: priyabalan8380@gmail.com

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ABSTRACT

Objective: An efficient ultra-high performance liquid chromatographic (UHPLC or Infinity LC 1290) method has been developed and validated for the quantification of possible carcinogenic or genotoxic impurities in febuxostat drug substances and drug products at 18 µg/ml level.

Methods: This method includes the conclusion of four potential genotoxic impurities in febuxostat. The mobile phase is trifluoroacetic acid, acetonitrile, and water with linear gradient elution. The UHPLC column used for the analysis was zorbax RRHD eclipse plus C18 with a length of 100 mm, internal diameter of 2.1 mm, and particle size of 1.8 µ.

Results: The limit of detection and limit of quantitation of the impurities are <0.1 (0.00001%) and 0.3 µg/ml (0.00003%) with respect to febuxostat test concentration of 1000 µg/ml, respectively. This method has been validated as per ICH guidelines Q2 (R1).

Conclusion: A rapid, cost-effective infinity LC method was wonderfully established for quantitative analysis of possible genotoxic impurities of febuxostat drug substance and drug products.

Keywords: Febuxostat, Genotoxic impurities, Ultra-high performance liquid chromatograph, Infinity-LC 1290, Validation.

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INTRODUCTION

Febuxostat is a drug that hinders xanthine oxidase, thus plummeting production of uric acid in the consistency. It is employed in the discussion of chronic gout and hyperuricemia. Febuxostat was revealed by scientists at the Japanese Pharmaceutical Company Teijin in 1998. Teijin united the drug with TAP Pharmaceuticals in the US and Ipsen in Europe. Ipsen obtained a marketing endorsement for febuxostat from the European medicines agency in April 2008, Takeda obtained FDA approval in February 2009, and Teijin attained sanction from the Japanese "Pharmaceuticals and Medical Devices Agency" in 2011. Febuxostat is used to treat chronic gout and hyperuricemia [1]. The National Institute for Health and Clinical Excellence concluded that febuxostat is extra effective than usual dosages of allopurinol, but not more effective than higher doses of allopurinol [1]. Febuxostat is in the US pregnancy category C; there are no adequate and well-controlled studies in pregnant women [2].

The determination of febuxostat and its related compounds were performed by LC studies at the level of 1000 µg/ml or 0.10% w/w with respect to the target analyte concentration. As per the selection of synthetic route, the related compounds of febuxostat may be categorized as genotoxic or carcinogenic. Nowadays, the regulators were very much interested about the genotoxic impurities present in drug substances and drug products. They assert to have a control of potential genotoxic impurities in the synthetic drug process. The control of potential genotoxic impurities shall be provided based on the availability of the high sensitive chromatography method. This directs us to evolve a method for the genotoxic impurities of febuxostat in the drug substances and drug products using ultra-high performance liquid chromatographic (UHPLC).

Other subjects were explained about the determination of febuxostat was performed by liquid chromatograph coupled mass spectrometer (LCMS/MS) in human plasma [3-6], by LC-ultraviolet (UV) [7], by HPLC [8-10], by UPLC [11-13], and by UPLC/MS in dog plasma [14]. The related compounds of febuxostat were separated and determined at the level 0.10% w/w level or 1000 µg/ml level by UPLC in literature [13]. Hence, as to increase the sensitivity of the method using these four genotoxic impurities in febuxostat, the research work has been initiated which was grounded in the literature [13,15-24,26-29].

As per the literature survey of the febuxostat drug substances and drug products, no one has reported the infinity LC/UHPLC method for the quantification of probable genotoxic impurities in febuxostat at 18 µg/ml level, and this is the novelty of the article.

Evaluation study of genotoxicity and carcinogenicity

From the appraisal study of genotoxic and carcinogenic impurities; imp-1, imp-2, imp-3, and imp-4 (Fig. 1) have been given an alert for potential carcinogen which was due to the presence of aldehyde functional group in its own structure of the molecule and also based on quantitative structure-activity relationship (QSAR). These alerts have been obtained from the Toxtree software and its version was 2.6.6. The recommended maximum daily dosage of febuxostat is about 80 mg. The threshold of toxicological concern limit could be 18.75 µg/ml as per the calculation provided in ICH guideline M7 and based on acceptable intake of 1.5 µg/day was considered to be protective for a lifetime of daily exposure.

METHODS

Instrumentation

An Agilent - 1290 series UHPLC/infinity LC consisting of a binary/quaternary pump, column compartment (Zorbax RRHD eclipse plus C18 with 100 mm × 2.1 mm, 1.8 µm was used as a column), autoinjector, and a diode array detector (M/s. Agilent Technologies, USA). Water bath equipped with controller (Amkette Analytics, ANM alliance) was used for forced degradation studies. Photolytic studies were carried out in a photostability chamber (Thermolab Photo Stability Chamber, India). Thermal degradation works were accomplished in a hot air oven (Amkette Analytics, ANM alliance).

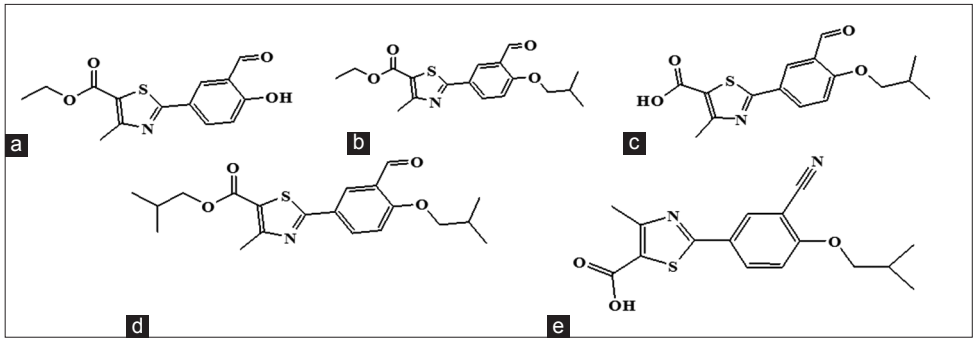

Fig. 1: The structure of potential genotoxic imurities and febuxostat. (a) ethyl 2-(3-formyl-4-hydroxyphenyl)-4-methylthiazole-5-carboxylate or imp-1, (b) ethyl 2-(3-formyl-4-isobutoxyphenyl)-4-methylthiazole-5-carboxylate or imp-2, (c) 2-(3-formyl-4-isobutoxyphenyl)-4-methylthiazole-5-carboxylic acid or imp-3, (d) isobutyl 2-(3-formyl-4-isobutoxyphenyl)-4-methylthiazole-5-carboxylate or im-4, (e) 2-(3-cyano-4-isobutoxy-phenyl)-4-methyl-thiazole-5-carboxylic acid or febuxostat

Reagents and chemicals

Febuxostat, imp-1, imp-2, imp-3, and imp-4 (Fig. 1) were synthesized in chemistry lab, St. Peter's university. Trifluoroacetic acid and acetonitrile were bought from Fisher Scientific. HPLC grade water was used, equipped with the Elga water purification system, Metrohm.

Solutions preparation

Sample diluent

The sample diluent was prepared by mixing 300 ml of mobile Phase A solution and 700 ml of mobile Phase B solution. Filtered and degassed for usage of analysis.

Standard solution

The standard solution was prepared by exactly weighed and transferred about 18 mg each of imp-1, imp-2, imp-3, imp-4 and febuxostat in 100 ml volumetric flask; added 50 ml of sample diluent and ultra-sonicated for 5 minutes. After sonication, the solution was made up to the mark with the sample diluent. Further, 10 ml of this solution was diluted into a 100 ml volumetric flask and made up to the mark with sample diluent. Hence, the standard solution concentration was 18 µg/ml with respect to the test concentration of 1000 µg/ml febuxostat. The solution was centrifuged at 25°C for 30 minutes and filtered through 0.22 µm syringe filter and injected. By keeping, the same solution at ambient temperature for 24 hrs and it was used for solution stability study.

Sample solution

The sample solution was prepared by accurately weighed and transferred about 10000 mg of sample into 10 ml volumetric flask; added 5 ml of sample diluent and ultra-sonicated for 5 minutes. After sonication, the solution was made up to the mark with the sample diluent. The solution was centrifuged at 25°C for 30 minutes and filtered through 0.22 µm syringe filter and injected. By keeping, the same solution at ambient temperature for 24 hrs and it was used for solution stability study.

Method development

Several methods have been developed by HPLC and UPLC for the determination of febuxostat in the bulk and formulated products. Some developed methods for the quantification of febuxostat and its related compounds in the bulk and formulated products by UPLC [3,8-14,26]. These previously published research articles were failed to explain about the probable genotoxic impurities in febuxostat. Actually, the four impurities of febuxostat, which were mentioned in Fig. 1, were evaluated as potential genotoxic impurities using QSAR guidelines and these impurities were determined at 1500 µg/ml level in febuxostat by UPLC as per the literature work.

This mainly leads us to do further development of the method with four genotoxic impurities in febuxostat at the 18 µg/ml level by UHPLC. The same method [13] has been used for the initial development, but the sensitivity of the method was found at the very lowest level of those impurities of the literature work [13]. Hence, as to increase the sensitivity of the method, the mobile phase, pH and gradient composition have been modified accordingly. The pH of the mobile phase was maintained in the acidic region based on the literature [13], which was achieved by the addition of 1 ml of trifluoroacetic acid in 1000 ml of water. The mass compatible mobile phase has been selected for the development and validation, because if any impurities were detected at the level of above or below limit of quantification (LOQ) in the sample which needs to be confirmed using UPLC-MS study.

Fortunately, these four potential genotoxic impurities were not present/not detected in the samples of drug substances and drug products. Hence, these samples were not screened by UPLC-MS. This could be the limitation of the present research work. These impurities have been eluted and separated well within the time of 6 minutes, but to ensure the consistency and specificity of the other impurities which was mentioned in the research article [26]; the gradient program has been slightly modified and extended up to 12 minutes. From this trial, the method was specific for all the impurities which have mentioned in the research article [26]. Furthermore, impurities of V and VI [26] were matching and other impurities were not matching with the present research work; hence, totally these four impurities considered for the research work. The results and comparisons of previously published articles were given in Table 1.

Concluded method for validation purpose

The UHPLC column was used Zorbax RRHD C18, 100 mm × 2.1 mm × 1.8 µm. The mobile Phase A was diluted 1 ml of trifluoroacetic acid in 1000 ml of water. The mobile Phase B was acetonitrile. The gradient program was mentioned as min/%B composition; 0.00/5.0, 0.80/5.0, 2.00/25.0, 2.40/35.0, 6.00/50.0, 6.40/65.0, 8.00/80.0, 9.00/90.0, 10.00/90.0, 10.20/5.0, and 12.00/5.0. The wavelength of detection was 320 nm and injection volume was 2 µl. The column compartment temperature was maintained at 45°C.

Method validation

Once chromatographic conditions were established; the method was validated in compliance with ICH guidelines. The following parameters of system suitability, specificity, linearity, precision, accuracy, limit of detection (LOD), LOQ, robustness, and forced degradation studies were performed for validation.

System suitability

The standard solution was prepared using standard as per the method. Moreover, six replicates are injected into the system.

Specificity

The specificity of the method was determined by comparing chromatograms obtained from standard, blank and that of forced degradation studies.

Table 1: Comparative of results with the previously published research works

References	Technique	Calibration procedure	Analyte	LLOD/LOD/MDL	LLOQ/LOQ/ MLQ	Sample preparation technique	Remarks
[3]	LCMS/MS	Internal standard	Febuxostat	0.5 pg/ml	1.00 ng/ml	Liquid-liquid extraction	Human blood serum
[8]	HPLC	External standard	Febuxostat	0.018 µg/ml	0.06 µg/ml	Dissolve-inject	Content
[9]	HPLC	External standard	Febuxostat	0.382 µg/ml	1.157 µg/ml	Dissolve-inject	Content
[10]	HPLC	External standard	Febuxostat	0.257 µg/ml	0.0783 µg/ml	Dissolve-inject	Content
[11]	UPLC	External standard	Febuxostat and its impurities	Di acid impurity=1.41 µg/ml Acid amide impurity=1.49 µg/ml N-propyl impurity=0.64 µg/ml Sec-butyl impurity=0.03 µg/ml Des cyano impurity=0.03 µg/ml Nitrile impurity=0.35 µg/ml	Di acid impurity= 4.26 µg/ml Acid amide impurity= 4.51 µg/ml N-propyl impurity= 1.93 µg/ml Sec-butyl impurity= 0.10 µg/ml Des cyano impurity= 0.08 µg/ml Nitrile impurity= 1.07 µg/ml	Dissolve-inject	Related compounds
[12]	UPLC	External standard	Febuxostat	0.15 µg/ml	1.2 µg/ml	Dissolve-inject	Content
[13]	UPLC	External standard	Febuxostat	Impurity A=0.14 µg/ml Impurity B=0.14 µg/ml Impurity C= 0.14 µg/ml ImpurityD= 0.14 µg/ml Febuxostat= 0.13 µg/ml	Impurity A= 0.42 µg/ml Impurity B= 0.42 µg/ml Impurity C= 0.42 µg/ml Impurity D= 0.42 µg/ml Febuxostat= 0.40 µg/ml	Dissolve-inject	Related compounds
[14]	UPLC-MS/ MS	NA	Febuxostat	NA	5-1000 ng/ml	Liquid-liquid extraction	Dog blood serum
Present work	Infinity LC	External standard	Potential genotoxic impurities	0.07 µg/ml	0.2 µg/ml	Dissolve - Centrifuge - inject	More sensitive method for drug substance and products

LCMS/MS: Liquid chromatograph coupled mass spectrometer, HPLC: High performance liquid chromatography, UPLC: Ultra performance liquid chromatograph, UPLCMS/MS: Ultra performance liquid chromatography coupled mass spectrometer

Linearity

The linearity of detector response was established by plotting a graph between concentrations versus average area responses of the analytes.

Precision

Six replicates injections of the standard solution were injected into the UHPLC system as considered as system precision. The precision of test method was evaluated by six preparations of spiked sample solutions with impurities of imp-1, imp-2, imp-3, and imp-4 from the same batch as considered as method precision.

Accuracy

A study of accuracy (recovery) was performed by spiking genotoxic impurities in drug substances and drug products. Samples were prepared as per the proposed method at QL-150% of the target test concentration.

LOD and LOQ limits

The LOD and LOQ were examined based on signal-to-noise ratio method as per the ICH guideline Q2 (R1). The signal to noise ratio for LOD is 3:1 and the LOQ is 10:1. This was performed by performing the sequence of dilute solutions with a known concentration LOD and LOQ has been determined.

Robustness

Robustness of the method was investigated by varying the instrumental conditions such as flow rate (±0.1 ml/min), column oven temperature (±5°C), and the addition of trifluoroacetic acid in mobile phase

(±0.2 ml). The standard solution was prepared and analyzed as per the test procedure monitored the system suitability results.

Forced degradation studies

Acid degradation studies

Accurately weighed and transferred about 50,000 mg of sample into a 50 ml of volumetric flask. Added 30 ml of diluent and 10 ml of 0.4 N HCl was added and refluxed at 60°C for 24 h and neutralized with 0.4 N NaOH. After the exposure, the resultant solution was made up to the mark with diluent. The solution was centrifuged at 25°C for 30 min and filtered through 0.22 µm syringe filter and injected into the system, and chromatograms were recorded to assess the stability of the sample.

Alkali degradation studies

Accurately weighed and transferred about 50,000 mg of sample into a 50 ml of volumetric flask. Added 30 ml of diluent and 10 ml of 0.4 N NaOH was added and refluxed at 60°C for 24 hrs and neutralized with 0.4 N HCl. After the exposure, the resultant solution was made up to the mark with diluent. The solution was centrifuged at 25°C for 30 minutes and filtered through 0.22 µm syringe filter and injected into the system, and chromatograms were recorded to assess the stability of the sample.

Oxidative degradation

Accurately weighed and transferred about 50,000 mg of sample into a 50 ml of volumetric flask. Added 30 ml of diluent and 10 ml of 3% H_2O_2 was added and kept at room temperature for 24 hrs. After the exposure, the resultant solution was made up to the mark with diluent. The solution was centrifuged at 25°C for 30 minutes and filtered through 0.22 µm syringe filter and injected into the system, and chromatograms were recorded to assess the stability of the sample.

Dry heat degradation

Accurately weighed and spread 10 g of sample in a dried Petri dish uniformly (maximum of 5 mm thickness). Placed the dish in an oven at 60°C for 7 days without vacuum. Recorded the weight (petri dish + sample) and noted the appearance before and after the stress test. Calculated percent change for the weight of sample. Weighed and transferred about 50,000 mg of stressed sample into a 50 ml of volumetric flask. After the exposure, the resultant solution was made up to the mark with diluent. The solution was centrifuged at 25°C for 30 minutes and filtered through 0.22 µm syringe filter and injected into the system, and chromatograms were recorded to assess the stability of the sample.

Photostability studies

Weighed and transferred about 50,000 mg of exposed sample into two 50 ml of quartz volumetric flasks and the solution was made up to the mark with diluent. One quartz volumetric flask was fully covered with aluminum foil and labeled as control sample solution, and another quartz volumetric flask was kept without covering aluminum foil and marked as stressed sample solution. Placed the tightly sealed flask in a suitable chamber and irradiate first under fluorescent light for a total exposure of 1.2×10^6 Lux hours, and then under UV fluorescent light for a total exposure of 200 W.hr/m^2. The solutions were centrifuged at 25°C for 30 minutes and filtered through 0.22 µm syringe filter and injected into the system, and chromatograms were recorded to assess the stability of the sample.

RESULTS AND DISCUSSION

Different chromatographic conditions were employed for the analysis of febuxostat in both bulk and pharmaceutical dosage forms. The comparative results of previously published articles were given in Table 1.

The standard solution was injected repeatedly and performed the calculation of % relative standard deviation (RSD) for each peak and values were obtained was <5%. This indicates that the equipment was accurate and fit for study. The system precision results have been given in Table 2. The Chromatographs of blank and standard have been shown in Fig. 2.

The %RSD for the content of imp-1, imp-2, imp-3, and imp-4 was obtained below 2%. The %RSD for content impurities was within 3% for intermediate precision which was performed by different analysts, column, instrument, and day. This result shows that the method was precise. Table 3 shows the results of method precision data.

The LOD for imp-1, imp-2, imp-3, imp-4 and febuxostat were 0.070, 0.076, 0.080, 0.070 and 0.073 µg/ml, respectively. The LOQ for imp-1,

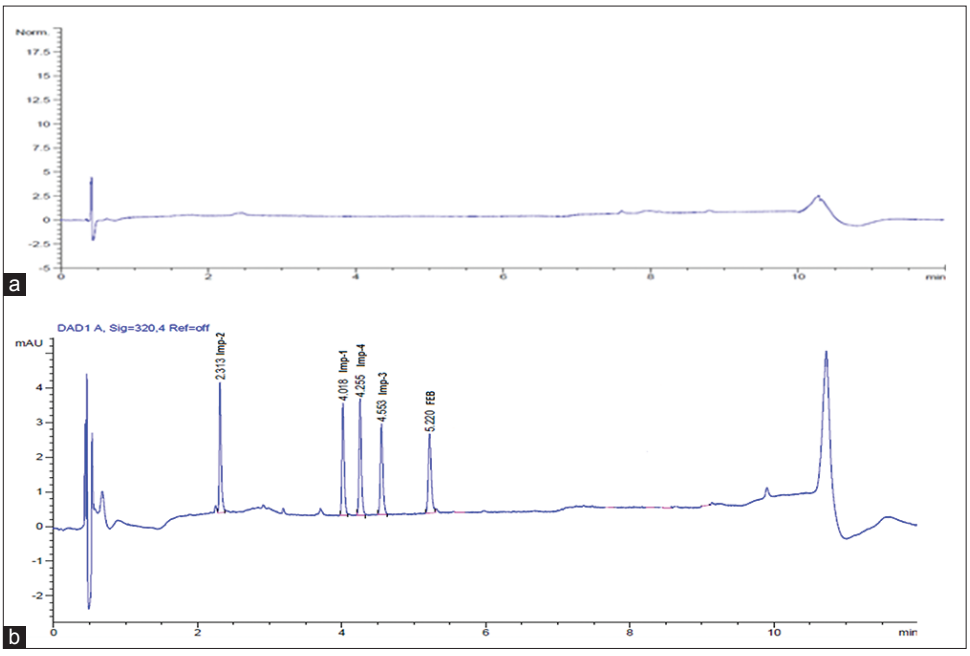

Fig. 2: (a and b) Chromatograms of blank and standard solution

imp-2, imp-3, imp-4 and febuxostat were 0.21, 0.23, 0.24, 0.21 and 0.22 µg/ml, respectively. The LOD and LOQ results indicated that the method was most sensitive when compared to the reported methods shown in Table 1.

The correlation coefficient values have been shown in Tables 4-8. The linearity graphs were shown in Figs. 3-7. The values of the correlation coefficients were almost equal to one; this indicates that the developed method was linear. The regression results indicate that the validated method was linear over the total concentration and it was satisfactory for its concentration range from LOQ to 150%. The percentage of bias was calculated by multiplying the slope with 100 and then the resultant was divided by the reference value, which can be considered as the area response obtained at the 100% level. The calculated % of bias values indicates that the method was linear and it was very close to the origin or close to the ideal theoretical value.

The accuracy percentage for imp-1, imp-2, imp-3 and imp-4 were 85-105% of drug substance and drug products. This result indicates that the method was accurate and appropriate as the mean accuracy value was within the limit (80-120%).

The specificity of the method was demonstrated by the peak purity (i.e., the purity angle is lesser than the purity threshold) using the diode array detector for degraded samples. The peak purity analysis was homogeneous for imp-1, imp-2, imp-3, and imp-4. There was no interference was observed from blank peaks and impurities. There was no secondary peak aroused from forced degraded samples. The results of forced degradation study indicate that the method was stability indicating and the impurities, *viz.*, imp-1, imp-2, imp-3 and imp-4 were not the degradation impurities, and these are process related impurities only.

By carefully varying in chromatographic conditions the resolution between febuxostat, imp-1, imp-2, imp-3 and imp-4 were evaluated. The resolution between impurities and febuxostat was obtained >1.8 in all the varied chromatographic conditions carried out (flow rate, the addition of trifluoroacetic acid and column temperature). The result of robustness analysis shows that the method was considered robust.

ACKNOWLEDGMENTS

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Table 2: System precision results of imp-1, imp-2, imp-3, imp-4 and febuxostat

Injection	Area observed				
	Imp-1	Imp-2	Imp-3	Imp-4	Febuxostat
1	145,204	109,528	92,163	97,921	65,121
2	144,956	110,785	93,001	97,810	68,265
3	145,060	111,527	92,964	97,777	69,842
4	145,329	112,328	92,763	97,707	70,798
5	145,239	111,833	93,521	97,917	71,827
6	145,110	112,045	92,661	97,811	72,287
Mean	145,150	111,341	92,846	97,824	69,690
SD	134.32	1033.69	447.48	82.85	2663.39
%RSD	0.09	0.93	0.48	0.08	3.82
Limit	RSD should be<15%				

Imp: Impurity, SD: Standard deviation, RSD: Relative standard deviation

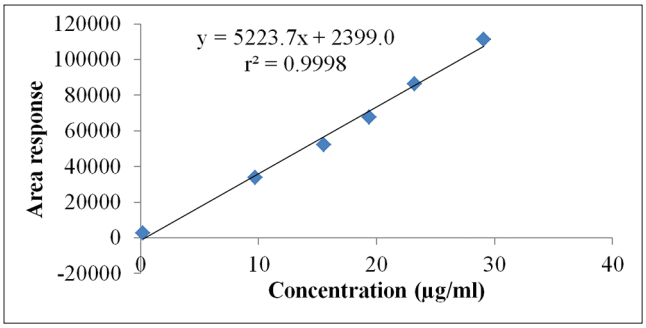

Fig. 3: Linearity graph for imp-1

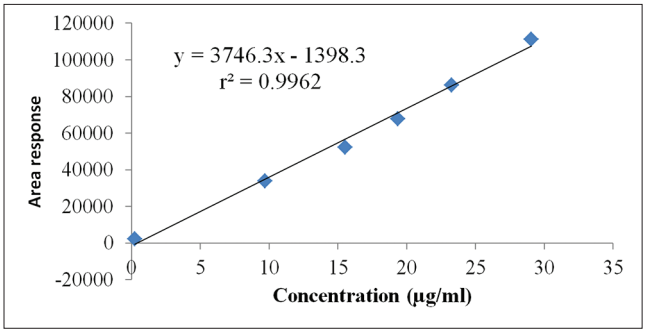

Fig. 4: Linearity graph for imp-2

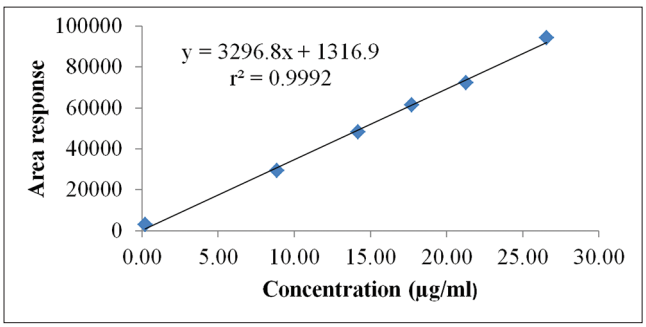

Fig. 5: Linearity graph for imp-3

Table 3: Method precision results of imp-1, imp-2, imp-3 and imp-4

Preparation	Imp-1 content (µg/ml)	Imp-2 content (µg/ml)	Imp-3 content (µg/ml)	Imp-4 content (µg/ml)
1	17.552	18.025	17.956	18.112
2	17.668	18.158	17.865	18.251
3	17.023	18.026	17.952	17.996
4	17.258	18.251	17.874	18.665
5	17.325	18.036	18.025	17.953
6	17.125	18.325	18.001	17.992
Mean	17.3	18.1	17.9	18.2
SD	0.25	0.13	0.07	0.27
%RSD	1.4	0.7	0.4	1.5
Limit	RSD for content should be<5%			

Imp: Impurity, SD: Standard deviation, RSD: Relative standard deviation

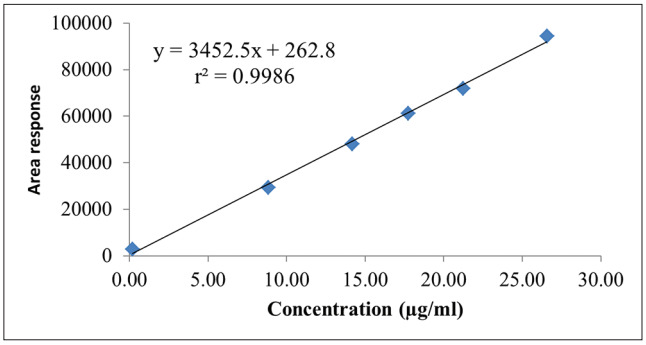

Fig. 6: Linearity graph for imp-4

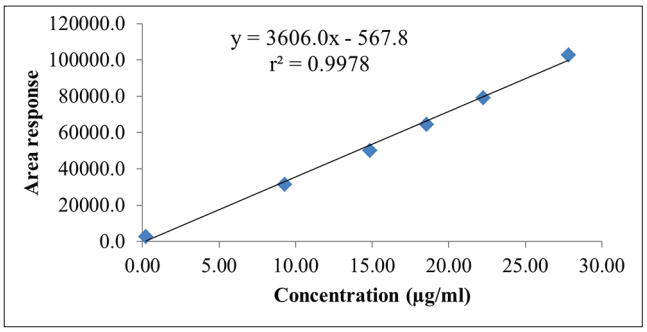

Fig. 7: Linearity graph for febuxostat

Table 4: Linearity data of imp-1

Sample	% Level	Concentration (µg/ml)	Peak response
1	LOQ	0.21	4385
2	50	9.0	48,745
3	80	14.5	77,380
4	100	18.1	96,529
5	120	21.6	115,521
6	150	27.2	145,150
Slope		5223.7	
Y-intercept		2399.0	
Correlation co-efficient square (r^2)		0.9998	
Bias (%)		5.4	

LOQ: Limit of quantification and each % level injected twice

Table 5: Linearity data of imp-2

Sample	% Level	Concentration (µg/ml)	Peak response
1	LOQ	0.23	2800
2	50	9.68	34,200
3	80	15.50	52,396
4	100	19.36	68,000
5	120	23.24	86,522
6	150	29.06	111,341
Slope		3746.3	
Y-intercept		−1398.3	
Correlation coefficient square (r^2)		0.9962	
Bias (%)		5.5	

LOQ: Limit of quantification and each level injected twice

CONCLUSION

A swift, economical infinity LC method was magnificently established for quantitative analysis of possible genotoxic impurities of febuxostat drug substance and drug products. The method was found to be specific and truthful with attired and constant recoveries. The valid method may be used for the regular analysis of the determination of potential genotoxic impurities of febuxostat drug substances and drug products in quality control laboratories.

Table 6: Linearity data of imp-3

Sample	% Level	Concentration (µg/ml)	Peak response
1	QL	0.24	3727
2	50	9.108	29,639
3	80	14.562	48,979
4	100	18.216	60,597
5	120	21.852	73,121
6	150	27.324	92,845
Slope		3296.8	
Y-intercept		1316.9	
Correlation coefficient square (r^2)		0.9992	
Bias (%)		5.4	

LOQ: Limit of quantification and each level injected twice

Table 7: Linearity data of imp-4

Sample	% Level	Concentration (µg/ml)	Peak response
1	QL	0.21	2890.5
2	50	8.86	29,183.6
3	80	14.16	48,165.6
4	100	17.71	61,301.2
5	120	21.25	72,133.0
6	150	26.57	94,285.9
Slope		3452.5	
Y-intercept		262.8	
Correlation coefficient square (r^2)		0.9986	
Bias (%)		5.6	

LOQ: Limit of quantification and each level injected twice

Table 8: Linearity data of febuxostat

Sample	% Level	Concentration (µg/ml)	Peak response
1	QL	0.22	2845.3
2	50	9.27	31,691.8
3	80	14.83	50,280.8
4	100	18.54	64,650.6
5	120	22.24	79,327.5
6	150	27.81	102,813.5
Slope		3606.0	
Y-intercept		−567.8	
Correlation coefficient square (r^2)		0.9978	
Bias (%)		5.6	

LOQ: Limit of quantification and each level injected twice

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