SYSTEMATIC LC-MS/MS METHOD FOR QUANTIFICATION OF 2,3-DIMETHYL-2H-INDAZOLE-6-AMINE CONTENT IN PAZOPANIB HYDROCHLORIDE

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ABSTRACT

A sensitive and rugged method was developed and validated by using liquid chromatography coupled with triple quadrupole spectrometer (LC/MS/MS) technique for the determination of 2,3-dimethyl-2H-indazole-6-amine content in pazopanib hydrochloride. The separation was achieved by using Hypersil BDS C18 15 cm *4.6 mm length and 5.0 μ m width column. Mobile phase-A was 0.1% formic acid in water and mobile phase-B was acetonitrile in the ratio of 45:55 v/v with 1.0 mL/min flow rate in isocratic mode. The limit of detection (LOD) and limit of quantification (LOQ) signal to noise ratio values were obtained as 3.7 and 10.9 respectively. The linearity range between 0.6 to 6.0 ng/mL and the correlation coefficient was found 0.999. The recovery found between 98.8 to 101.3% at four different levels.

Keywords: Genotoxic Impurity, Pazopanib Hydrochloride, ICH, LC/MS/MS, LOD, LOQ.

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INTRODUCTION

Pazopanib hydrochloride is a tyrosine kinase inhibitor and used for the treatment of hypersensitivity.¹ The chemical name of pazopanib hydrochloride is 5-[[4-[2,3-dimethyl-2H-indazole-6-yl) methyl amino]-2-pyrimidinyl]amino]-2-methylbenzenesulfonamide with HCl salt. The molecular formula of pazopanib hydrochloride is $C_{12}H_{23}N_7O_2S.HCl$ and molecular weight about 473.99 grams per mole. Pazopanib hydrochloride available in slightly yellow solid, practically insoluble above pH 4 and very slightly soluble at pH 1.2,3-dimethyl-6-amino-2H-indazole-6-amine is used in the earlier-stage synthesis of pazopanib hydrochloride. The molecular formula of 2,3-dimethyl-2H-indazole-6-amine is $C_9H_{11}N_3$ and molecular weight about 161.21 grams per mole. The chemical structure of 2,3-dimethyl-2H-indazole-6-amine and pazopanib hydrochloride was shown in Fig.-1.

Fig.-1: Chemical Structure of 2,3-Dimethyl-2H-indazole-6-amine and Pazopanib Hydrochloride

Pharmaceutical impurities² are categorized into three types, they are inorganic, organic and residual solvents, those are known and unknown impurities and found during the manufacturing process of chromatographic techniques like TLC, HPLC, GC and LC/MS/MS.

2,3-dimethyl-2H-indazole-6-amine is a potential genotoxic impurity. European and ICH (International Council for harmonisation) M7 regulatory bodies expected the limit of genotoxic impurity³⁻⁴ should be below 2 ppm. There are many articles published by using LC/MS/MS technique.⁵⁻⁷ Usually, the term genotoxicity used in studies of in-vivo and in-vitro to find the genetic modification in genes by using a different mechanism.

EXPERIMENTAL

Chemical and Reagents

All chemicals were used above 99% purity. 2,3-Dimethyl-2H-indazole-6-amine impurity and pazopanib hydrochloride were procured from the perfomics analytical lab, Hyderabad. The formic acid, LCMS grade acetonitrile, HPLC grade water were got from the Merck life sciences.

Instrumentation

Liquid chromatography coupled with triple quadrupole is a powerful tool used extensively in the pharmaceutical and biopharmaceutical industry. Applied biosystem sciex QTRAP (Model: 4000) mass spectrometer and waters HPLC (Model: e2695) was used to carry out the analysis. Mettler Toledo (Model: XP56) microbalance was used to weigh the samples. Analyst 1.7 software on dell computer (Digital Equipment Co) used for the integration and quantification of chromatographic peaks.

Chromatographic Conditions

During the method validation “Hypersil BDS C18 (15 cm x 4.6 mm, 5.0 μ m)” column was used which is manufactured by Thermo fisher scientific. The mobile phase constitutes solution-A and solution-B Solution-A was 0.1% formic acid in water and solution-B was acetonitrile in the ratio of 45:55 (solution-A: solution-B, v/v). The injection volume 20 μ L with a flow rate of 1.00 mL/min. The program was used isocratic mode with a shorter run time of 5 minutes.

Mass Spectro Meter Parameters

The optimised parameters were temperature: 450°C, Ion spray voltage: 5500.00, Curtain gas: 40.0, DP: 51.0 volts, EP: 15.0 volts, CE: 27.0 volts, CXP: 16.0 volts GS1: 50.0 GS2: 40.0, CAD GAS: 8, split ratio: 4:6 for 2,3-Dimethyl-2H-indazole-6-amine. Positive MRM mode was used with m/z values 162.0 >147.0 for 2,3-dimethyl-2H-indazole-6-amine and the details of the respective mass spectrum of m/z values were shown in Fig.-2 and Fig.-3.

Fig.-2: Q1 Mass Spectrum of 2,3-Dimethyl-2H-indazole-6-amine

Fig.-3: Q3 Mass Spectrum of 2,3-Dimethyl-2H-indazole-6-amine

Standard and Sample Preparation

Water and acetonitrile in the ratio 40:60 v/v used as a diluent. The standard stock solution was prepared by weighed about 10 mg of 2,3-dimethyl-2H-indazole-6-amine standard into 100 mL and further 0.6, 1.0, 1.5, 2.0, 4.0 and 6 ppm concentrations samples were prepared from standard stock solution with respect to test concentration. The sample solution was prepared 1 mg/mL with diluent.

RESULTS AND DISCUSSIONS

Method Development

During the method development, many trials were performed by using the HPLC technique with different types of buffers, solvents, columns in isocratic and gradient mode. All trials were ineffectiveness to get the 6 ppm sensitivity due to elution of unknown impurities along with desired 2,3-dimethyl-2H-indazole-6-amine impurity, hence method was the switchover from the HPLC to LC/MS/MS technique to get the 6 ppm sensitivity. Different types of buffers, solvents, columns were used while performing the method development by using LC/MS/MS as a trial and error method. Finally the separation of 2,3-dimethyl-2H-indazole-6-amine and pazopanib hydrochloride by using Hypersil BDS C18 15 cm*4.6 mm length and 5.0 μ m width column. The mobile phase was solution-A containing 0.1% formic acid and solution-B acetonitrile in the ratio of 45:55 v/v with a flow rate of 1.0 mL/min in isocratic mode. The column oven temperature was 25°C; the sample cooler temperature was 20°C and mass spectrometer parameters optimized to get maximum sensitivity.

Method Validation

Specificity

Initiated the method validation⁸ with specificity parameter by injecting diluent, 2,3-dimethyl-2H-indazole-6-amine and pazopanib HCl solutions. It was observed that there is no interference observed at the retention time of the 2,3-dimethyl-2H-indazole-6-amine impurity and pazopanib HCl. The corresponding specificity chromatograms were shown in Fig.-4, Fig.-5 and Fig.-6 respectively.

System Suitability

As a part of the system suitability for checking the system was established by injecting the six replicate injections of limit level (2.00 ppm) standard solution and found the % RSD as 0.7% and details tabulated in Table-1.

Table-1: System Suitability of 2,3-Dimethyl-2H-indazole-6-amine.

Injection No.	Area Counts of 2,3-Dimethyl-2H-indazole-6-amine
1	117569
2	118568

3	117041
4	119121
5	118436
6	117096
Aveg.area	117972
Std. dev.	858.8
%RSD	0.7

Fig.-4: Blank Chromatogram of Specificity

Fig.-5: Specificity Chromatogram for 2,3-Dimethyl-2H-indazole-6-amine

Fig.-6: Specificity Chromatogram of Pazopanib Hydrochloride

Determination of Limit of Detection and Limit of Quantification

These two parameters play an important role in method validation. By injecting 0.2 ppm and 0.6 ppm of LOD and LOQ individual solutions of 2,3-dimethyl-2H-indazole-6-amine each with respect to the drug substance concentration of 1 mg/mL and determined their S/N ratios and obtained S/N values were 3.7 for LOD and 10.9 for LOQ.

Linearity

The linearity of 2,3-dimethyl-2H-indazole-6-amine was acceptable with a six-point calibration curve 0.6, 1.0, 1.5, 2.0, 4.0 and 6.0 ppm concentrations. The slope, intercept, and correlation coefficient values were found from linear least squares regression analysis. The correlation coefficient obtained was >0.999 and shown in Fig.-7. The corresponding linearity data was presented in Table-2.

Table-2: Linearity Table of 2,3-Dimethyl-2H-indazole-6-amine

Sample Name	Number of Values used	Mean (Peak area)	Expected Concentration (ppm)	Standard Deviation	%RSD
LOQ (0.6 ppm)	1 of 6	31681	0.63	504.2	1.6
L1 Solution (1.0 ppm)	1 of 3	62104	1.049	273.6	0.4
L2 Solution (1.5 ppm)	1 of 3	92221	1.574	663.1	0.7
L3 Solution (2.0 ppm)	1 of 3	119148	2.099	365.1	0.3
L4 solution (4.0 ppm)	1 of 3	236560	4.198	339.1	0.3
L5 Solution (6.0 ppm)	1 of 6	344188	6.296	339.2	0.1

Slope: 54668
Intercept: 3273
 $R^2=0.9990$

Fig.-7: Linearity Plot of 2,3-Dimethyl-2H-indazole-6-amine.

Precision

The method precision was performed by calculating the %RSD of six individual preparations by injecting six freshly prepared solutions covering limit (2 ppm) spiked with the sample solution. The intermediate precision was performed in different instruments on different days. The results were shown in Table-3. %RSD values were lower than 2.0% for both studies; these results confirmed an acceptable precision of the method development.

Accuracy

The recovery studies were performed from LOQ to 400%, where the recovery was checked for LOQ level, L1 level (50%) and L4 level (400%) in triplicate injection and L2 level (100%) in six replicate injections. The recovery was obtained for 2,3-dimethyl-2H-indazole-6-amine was between 98.8 to 101.3%, which indicates the method was accurate. The accuracy at LOQ chromatogram as shown in Fig.-

8 and the recovery values and %RSD were shown in Table-4.

Table-3: Intra-day and Inter-day Precision of 2,3-Dimethyl-2H-indazole-6-amine.

Sample ID	Method precision		Ruggedness (Intermediate precision)	
	Recovery (ppm)	Recovery (%)	Recovery (ppm)	Recovery (%)
1	1.912	95.3	2.016	100.3
2	1.99	99.2	2.031	101
3	1.913	95.3	1.996	99.3
4	1.988	98.8	2.047	101.8
5	1.965	97.9	2.014	100.2
6	1.967	98	1.991	99.1
Average	1.956	97.4	2.016	100.3
Std. dev.	0.04	1.71	0.02	1.02
% RSD	1.8	1.8	1	1

Fig.-8: Accuracy Chromatogram at LOQ Level

Robustness

The robustness was defined as the repeatability of the method shall be the same when slight changes happen in the method parameters; hence the flow rate was altered by 0.1 units from 1.1 mL/min to 0.9 mL/min and temperature changes from 23°C to 27°C. The %RSD was observed less than 1% in all conditions and results were shown in Table-5 and Table-6.

Table-4: The Recovery of 2,3-Dimethyl-2H-indazole-6-amine at Four Different Concentrations

Theoretical Conc. (ppm)	Measured Conc. (ppm)	Recovery %	%RSD
0.6	0.5984	99.6	0.7
0.6	0.6001	100.1	
0.6	0.6022	100.3	
1	1.0122	101.1	0.8
1	1.0141	101.3	
1	0.9988	99.9	
2	1.9946	98.8	0.7
2	1.9981	99.3	
2	1.999	99.7	
2	2.0069	100.2	
2	2.0008	100.1	
2	2.0018	100.4	
6	6.0102	100.2	0.6
6	6.0081	100.1	
6	5.9923	99.9	

Table-5: Robustness Data for Flow Rate Changes

Injection No.	Actual Flow (1.00 mL/min)	Low Flow (0.90 mL/min)	High Flow (1.10 mL/min)
1	117569	119412	116406
2	118568	119418	115638
3	117041	118631	116931
4	119121	118899	115463
5	118436	119560	116099
6	117096	118069	114011
Avg. area	117972	118998	115758
Std. dev.	858.8	578.1	1006.5
% RSD	0.7	0.5	0.9

Table-6: Robustness Data for Column Temperature Changes

Injection No	Actual Column Temp (25°C)	Low Column Temp (23°C)	High Column Temp (27°C)
1	117569	118544	118238
2	118568	119056	119756
3	117041	119985	118987
4	119121	118406	118874
5	118436	118963	118696
6	117096	118047	118966
Avg. area	117972	118834	118920
Std. dev.	858.8	674.9	494.6
% RSD	0.7	0.6	0.4

CONCLUSION

A simple and rugged method was developed for the determination of trace level 2,3-dimethyl-2H-indazole-6-amine impurity in Pazopanib Hydrochloride using LC-MS/MS. Results obtained during the method validation were precise, accurate and S/N ratio for the LOD:3.7 and LOQ:10.9 with respect to acceptable values. This method can be used in further API analysis to identify and quantify the 2,3-dimethyl-2H-indazole-6-amine impurity in Pazopanib hydrochloride manufacturing.

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