Original Article

Identification, Isolation And Characterization Of Unknown Potential Genotoxic Impurities In Silodosin

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Abstract

Two unknown impurities were detected during the related substance analysis of the Silodosin crude sample using High-Performance Liquid Chromatography. These impurities were isolated using preparative HPLC and then subjected to LCMS for mass determination. Structural elucidation has been carried out using NMR (^{1}H NMR, ^{13}C NMR) spectroscopy. These impurities were discovered to have a structural alert, which was confirmed by the Insilico prediction model indicating that they were Potentially Genotoxic Impurities (PGI's). A genotoxic assessment of these impurities was done using the Insilico prediction model for genotoxicity (VEGA and Toxtree). These impurities have not been reported elsewhere, to the best of our knowledge. Thus, a HPLC-UV method was developed and validated for the determination of these impurities at the TTC level. The proposed method was validated in terms of Specificity, Precision, Accuracy, Linearity and Robustness as per ICH guidelines. The LOD and LOQ of the method were established to be 0.0018 ppm and 0.055 ppm with respect to test concentration (1000 µg mL^{-1} silodosin).

Keywords: HPLC, Impurity Profiling, Genotoxic impurities, Silodosin, LCMS-NMR

1. INTRODUCTION

Silodosin is a chemical entity used to cure Benign prostatic hyperplasia (BPH). It is also known as (1-(3-hydroxypropyl)-5-[(2R) -(2-[2-[2-(2,2-trifluoroethoxy) phenoxy] ethyl amino) propyl] indoline-7-carboxamide. It has a high uroselectivity as an α1-adrenoceptor antagonist. Silodosin is a drug used to treat symptoms associated with an enlarged prostate such as difficulty urinating, urinary frequency and urgency in men. Silodosin belongs to the alpha-blocker group of medications. It alleviates BPH symptoms by relaxing the bladder and prostate muscles. The molecular structure of silodosin is shown in Fig. 1. Literature surveys reveal that very few methods available for impurity profiling of silodosin. It includes the estimation of silodosin and its metabolites in biological matrices [1]. A ultra-performance liquid chromatography (UPLC) method for estimating silodosin impurities and a Liquid Chromatography-FTMS method for characterizing silodosin degradant products were developed [2, 3].

As the effect of the medicine is inextricably connected to human health, regulatory guidelines are becoming more stringent. According to the ICH Q3B guideline, any impurity above 0.1% level in drug substances needs to be identified. If an impurity has a structural alarm, it must be examined for genotoxicity. The identification of

genotoxicity hazards is part of the impurity qualification procedure for drug substances and products. Genotoxicity of impurity can be assessed using Insilico prediction of genotoxicity or by performing the Ames bacterial mutagenicity test. In the absence of sufficient safety experimental data, the structural alert is used as a sign to differentiate the routine impurities and carcinogenic impurities in EMA, FDA and ICH guidelines [4].

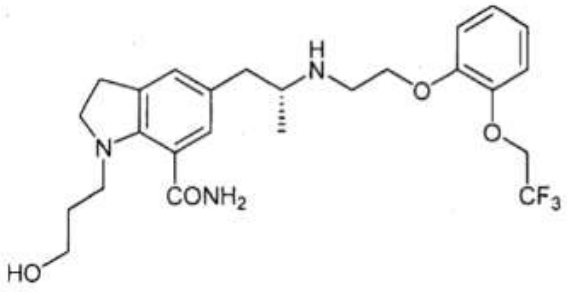

Fig. 1: Structure of silodosin.

Computational (Insilico) approaches may be used to investigate the mechanism and mode of action behind chemical toxicity and carcinogenicity, as well as to reduce laboratory experimentation. "Computational Predictive toxicology methods are accepted worldwide for the regulatory qualification of impurities, contaminants and degradant in pharmaceuticals" [5, 6]. According to the proposed ICH M7 guideline, "the computational assessment for the qualification of impurities in pharmaceuticals should be performed using an expert rule-based method and a secondary statistically-based QSAR model" [7].

In this work, we have used two computational assessment software for the prediction of toxicology and carcinogenicity, which are Toxtree (expert rule-based) and VEGA (statistical rule-based). Toxtree is an expert rule-based programme that categorizes chemicals and predicts various types of toxic effects using a decision tree approach. Toxtree provides a systematic toxicity classification approach (Cramer scheme and extended Cramer scheme), along with mutagenicity and carcinogenicity (ToxMic rule based on the in vivo micronucleus assay and the Benigni-Bossa rule base). VEGA is a statistically based model that was developed with the help of regulators and public entities in Europe and the United States. VEGA's initial nucleus is derived from the CAESAR model, which is comprised of a set of statistically-based models developed as part of the EU-funded CAESAR project. Prediction for numerous endpoints such as mutagenicity (AMES), carcinogenicity, bioconcentration factor, developmental toxicity, skin sensation, etc., may be produced. VEGA uses four distinctive QSAR models for the prediction of mutagenicity (namely CAESAR, ISS, SarPy and KNN) while for the prediction of carcinogenicity, it offers four models (ISS, CAESAR, IRFMN/ISSCAN-CGX and IRFMN/ANTARES).

Genotoxic impurities are those that have been shown to be genotoxic in a suitable genotoxicity test model, such as the AMES mutagenicity test. "Potential Genotoxic Impurities (PGIs) are those that have structural alert related to mutagenicity but that has not been tested in the experimental test model" [8, 9]. In this work, we demonstrated how we uncovered two new impurities in silodosin, and how, following characterization, it was found that these impurities had a structural alert, and how, using an Insilico Prediction model, it was proven that these impurities are potentially genotoxic. As per the ICH M7 guideline, these impurities need to be controlled at the TTC (Threshold of Toxicological Concern) level. The TTC concept provides an estimation of safe exposure for any mutagenic compound [7, 10]. "A TTC-based acceptable intake of 1.5 µg/day per person is thought to pose a negligible risk" [7, 11]. Based on TTC, for silodosin with a maximum daily dosage of 0.0081 g, the permitted level of PGIs in silodosin is 185 ppm (1.5 µg /0.0081 g).

No PGIs in silodosin have been reported in a literature review, and no analytical technique has been reported either. A rapid and simple HPLC-UV method was developed for simultaneous determination of both the impurities at the genotoxic level. This method was used to analyse silodosin for its PGIs at LOQ level of 0.055 ppm and LOD level of 0.0185 ppm w.r.t analyte concentration (1000 µg mL^{-1}). The method is linear across a 0.005-0.28 ppm range. The recoveries were found good over the range between 95-105%.

2. EXPERIMENTAL

2.1. Materials and Reagents

Sample of Silodosin API was received from IPCA Laboratories Ltd, HPLC grade Methanol, Acetonitrile, Ortho Phosphoric acid (88%) and Potassium dihydrogen phosphate were purchased from Merck, India. Milli Q water, obtained from Millipore India, was used throughout the analysis. Chloroform ($CDCl_3$) for NMR was purchased from Aldrich Chemicals Co., USA.

2.2. Mobile phase preparation

The mobile phase A is comprised of 0.01 M phosphate buffer, the pH of which is adjusted to 2.40 with orthophosphoric acid and filtered through 0.45µ filter paper. Acetonitrile is used as mobile phase B. After being degassed, both mobile phase solutions were preserved at ambient room temperature for further use.

2.3. High Performance Liquid Chromatography

The samples were analysed using a Waters Alliance 2695 HPLC system configured with a 2489 UV detector [Waters Corporation, USA] [12]. A Phenomenex Gemini NX C18 column (250mm x 4.6mm, 5µm) was employed for separation. The mobile phase flow rate was fixed at 0.7 mL min^{-1}. The gradient programme was established as T/%B: 0/20, 5/20, 25/50, 50/60, 55/60, 60/20, and 70/20. A constant temperature of 30°C was maintained inside the column. The detector wavelength was tuned to 225 nm, and the injection volume was adjusted to 30µl. The diluent used for sample preparation was, 0.1 % OPA (88%) in water and acetonitrile in a ratio of 80:20.

2.4. Preparation of standard and sample solutions

A stock mixture of Silodosin PGIs (Impurity 1 and Impurity 2) having a concentration of 0.1 mg mL^{-1} was prepared. From this stock solution, a standard solution containing 0.185 µg mL^{-1} of PGIs was prepared. (0.0185% w.r.t test concentration). A test preparation of 1000ppm of silodosin crude sample was prepared by dissolving 25 mg of silodosin in a 25 mL volumetric flask. For LOD, LOQ and Linearity a solution ranging from 0.0018 ppm to 0.28 ppm was prepared. For the Accuracy study, LOQ to 150% PGIs (Impurity 1 and Impurity 2) was spiked in a 1000ppm silodosin crude sample.

2.5. Preparative Liquid Chromatography

To isolate impurities from the crude sample, a Waters Alliance 2695 Separation module configured with a Waters 2489 UV PDA detector (Waters, USA) was employed. A Phenomenex Luna C18 (250mm x 21.2mm x particle size, 5µm) used, for, separation., Mobile phase A comprises of 0.1%, ammonium acetate and mobile phase B comprises of acetonitrile. UV detection was accomplished at 225 nm with a flow rate of 21 mL min^{-1}.

2.6. Liquid Chromatography Mass Spectroscopy (LC/MS)

The LCMS/MS studies were carried out using a Q-Exactive Orbit trap mass spectrometer from Thermo Fisher Scientific. For ionization, a Heated electron spray ionization (HESI) source was used. The spray voltage was upheld at 4.0 kV, the capillary temperature was fixed at 320°C and the auxiliary gas flow rate was kept at 5. Nitrogen was employed as a sheath and as an auxiliary gas. The heater temperature was held at 250°C, while the S-lens RF level was fixed at 100. With positive ion polarity, the mass to charge ratio (m/z) was examined at 70,000 resolution within a range of 100 to 1000 amu. An Agilent-1100 series quaternary gradient pump with a degasser, a column oven, and an autosampler were used in the HPLC. For separation, a C18 column (Phenomenex Gemini NX C18 column 250 mm x 4.6 mm i.d. 5 µm) was used. The mobile phase consists of A: 0.1 % ammonium acetate and B: Acetonitrile with time gradient programme T min/A: B: T0/90:10, T5/90:10, T35/20:80, T40/20:80, T42/90:10, T50/90:10.

2.7. NMR Spectroscopy

The isolated impurities ^{1}H and ^{13}C NMR spectra were measured on the ADVANCE 400 instrument (Bruker, Fallanden, Switzerland). The chemical shift measurement for ^{1}H and ^{13}C were given in δ scale (ppm) relative to $CDCl_3$.

3. RESULT AND DISCUSSION

3.1. Detection of unknown impurities

During HPLC analysis of the silodosin sample, two unknown impurities were detected at RRT 0.51 and 2.90 related to the principal peak. The typical crude silodosin chromatogram indicating the retention of impurities and the synthesis scheme of silodosin are shown in Fig. 2 and Fig. 3, respectively.

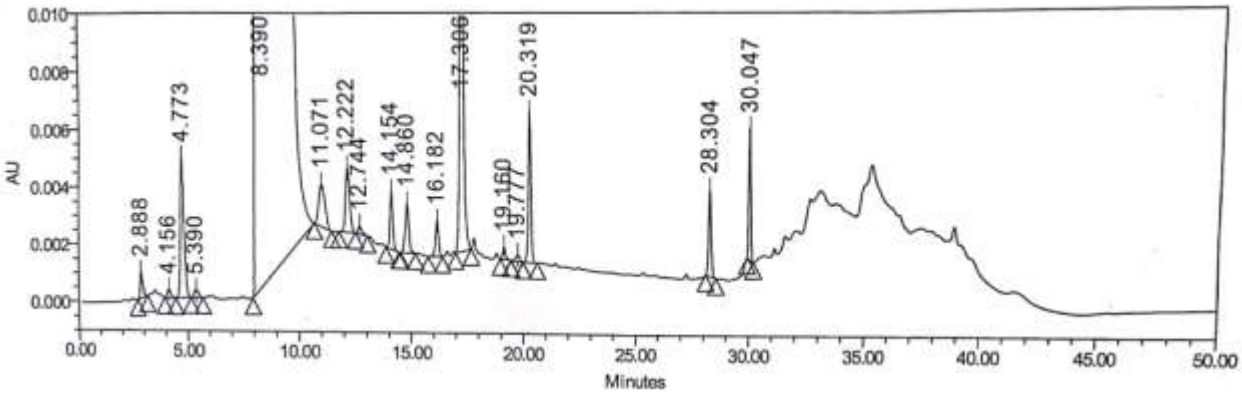

Fig. 2: Typical chromatogram showing Impurity 1 at RT about 4.773 and Impurity 2 at RT about 30.047 in crude sample of silodosin.

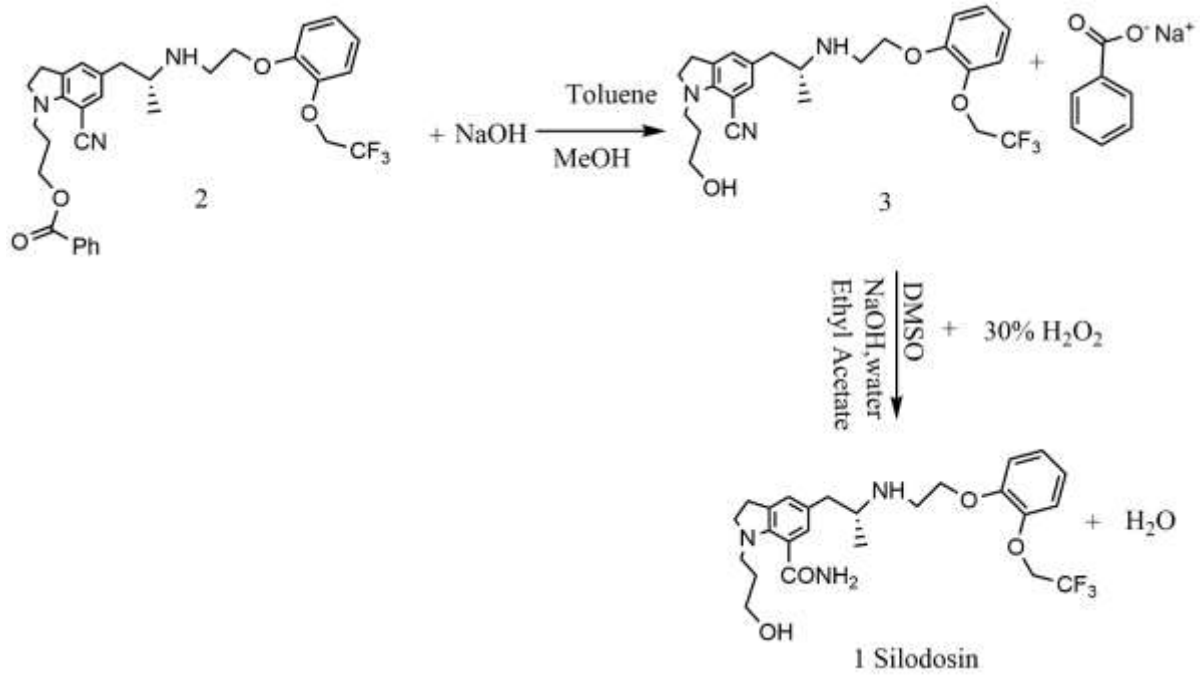

Fig. 3: Synthesis scheme of Silodosin.

3.2. Isolation and identification of impurities

The detected impurities were isolated using preparative HPLC. It was necessary to determine the mass, as impurities were unknown. Hence, the impurities were subjected to LCMS for mass determination. Fig. 4 and Fig. 5 shows the Liquid Chromatography Mass Spectroscopy spectra's of Impurity 1 and Impurity 2, respectively. Impurity 1 exhibited a protonated molecular ion peak $[M+H]^+$ at 260 having a molecular mass of 259 (Fig. 4). Impurity 2 exhibited a protonated molecular ion peak $[M+H]^+$ at 696 having a molecular mass of 695 (Fig. 5).

Fig. 4: Liquid Chromatography Mass Spectroscopy spectrum of Impurity 1.

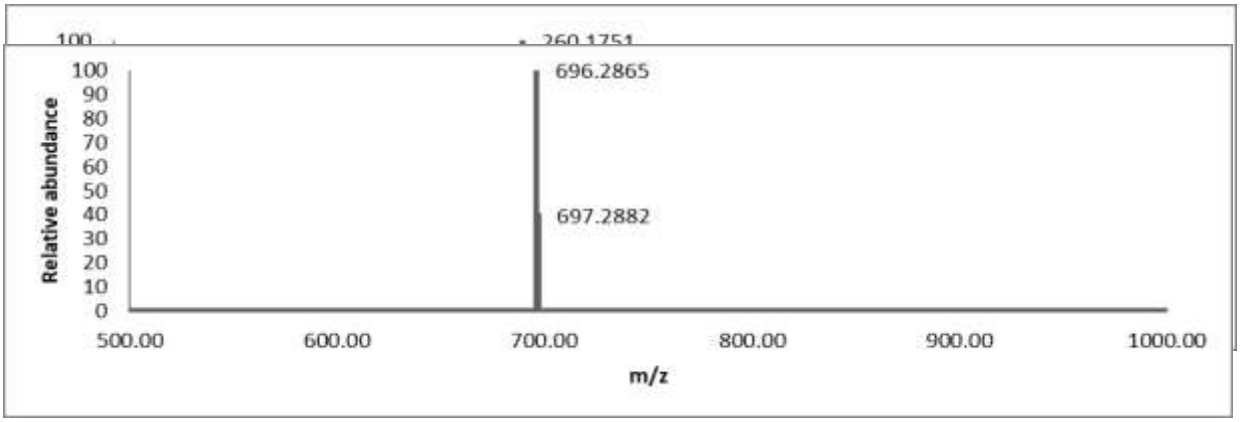

Fig. 5: Liquid Chromatography Mass Spectroscopy spectrum of Impurity 2.

3.3. Characterization and Genotoxic assessment of impurities

The unknown impurity formed during the synthesis of silodosin was isolated using preparative HPLC as described above. Comparative ^{1}H-NMR assignment of Impurity1 and Impurity 2 is tabulated in Table 1. Comparative ^{13}C NMR assignment data of Impurity 1 and Impurity 2 are mentioned in Table 2. The chromatographic purity was tested and determined to be around 99%. ^{13}C NMR and ^{1}H spectral data confirmed the structure. The structures of Impurity 1 and Impurity 2 are depicted in Fig. 6 and Fig.7. Based on the LCMS and NMR data the molecular formula of the Impurity 1 is confirmed as $C_{15}H_{21}N_3O$ (Fig. 6) and Impurity 2 as $C_{35}H_{39}F_6N_3O_5$ (Fig. 7). These two new impurities were designated as 1-(3-hydroxypropyl)-5-[(2R)-{2bis[2[2(2,2,2trifluoroethoxy)phenoxy]ethyl}amino)propyl)indoline-7-carbonitrile (Impurity1) and 5-[(2R)-2-aminopropyl]-2,3-dihydro-1-(3-hydroxypropyl)-1H-Indole-7-carbonitrile (Impurity 2). The characterization result alarmed the presence of a genotoxic functional group. The nitrile group present in both impurities falls under Cramer's class III substance which suggests significant toxicity [13]. Thus, a genotoxic assessment of impurities was performed using the Toxtree and VEGA software. Prediction reports from both the tools confirmed the mutagenicity as well as carcinogenicity of the impurities.

Table 1: Comparative ^{1}H-NMR assignment of Impurity1 and Impurity 2

Position	**Impurity-1, δ ppm, multiplicity**	**Impurity-2, δ ppm, multiplicity**
1	1.88-1.89, 2H, m	1.03-1.05, 3H, d
2	3.05, 1H, m	3.06, 1H, m
3	1.09, 3H, d	2.33 & 2.70, Ha & Hb, m
4	2.51 & 2.95, Ha & Hb, m	-
5	-	6.98, 1H, m
6	6.94, 1H, s	-
11	-	7.03, 1H, m
12	6.96, 1H, s	2.83, 2H, m

13	3.00, 2H, t	3.50-3.54, 2H, t
14	3.57, 2H, t	-
15	-	3.63-3.67, 2H, m
16	3.78, 2H, t	1.90, 2H, m
17	1.91-1.93, 2H, m	3.80-3.83, 2H, t
18	3.66, 2H, m	2.03, 1H, brs
19	1.94, 1H, m	-
20,35	-	2.83-3.04, 4H, m
21,36	-	3.95-4.04, 4H, m
24,27	-	6.93, 2H, m
25,26	-	6.88, 2H, m
30,45	-	4.33-4.40, 4H, m
39,42	-	6.98, 2H, m
40,41	-	6.91, 2H, m

s– singlet; m – multiplet; d – doublet; t – triplet; brs – broad singlet; q - quartet

Table 2: Comparative ^{13}C NMR assignment Impurity 1 and Impurity 2

Position	**Impurity-1, δ ppm**	**Impurity-2, δ ppm**	**Position**	**Impurity-2, δ ppm**
1	-	15.0	21	68.1
2	45.2	60.5	23	147.2
3	23.3	39.0	24	125.0
4	45.2	129.7	25	149.7
5	128.1	129.2	26	149.7
6	131.3	87.4	27	125.0
7	87.6	117.7	28	147.2
8	119.9	-	30	113.9
9	-	132.5	31	122.2
10	152.0	151.7	35	50.2
11	129.7	131.1	36	68.1
12	132.8	27.2	38	147.2
13	27.4	67.7	39	125.0
14	53.3	-	40	149.7
15	-	45.7	41	149.7
16	48.3	30.5	42	125.0
17	30.5	59.3	43	147.2
18	60.2	-	45	113.9
20	-	53.4	46	122.2

3.4. Formation of Impurity 1 and Impurity 2

Figure 8 shows the reaction scheme of formation of Impurity 1 and Impurity 2 of Silodosin. Impurity-2 is a process-related impurity which is formed as a side product sometime, it happens to form as a result of dimerization of (2,2,2-trifluoroethoxy) benzene group at the nitrogen site while Impurity-1 formed as a result of hydrolysis. Its formation could be prevented by controlling hydrolysis conditions.

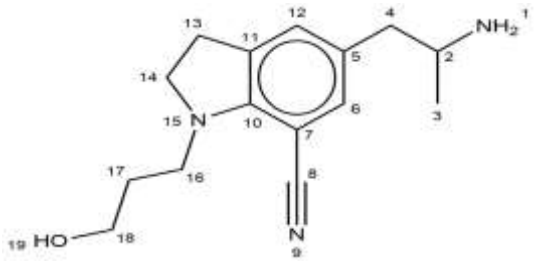

Fig. 6: Structure of Impurity 1.

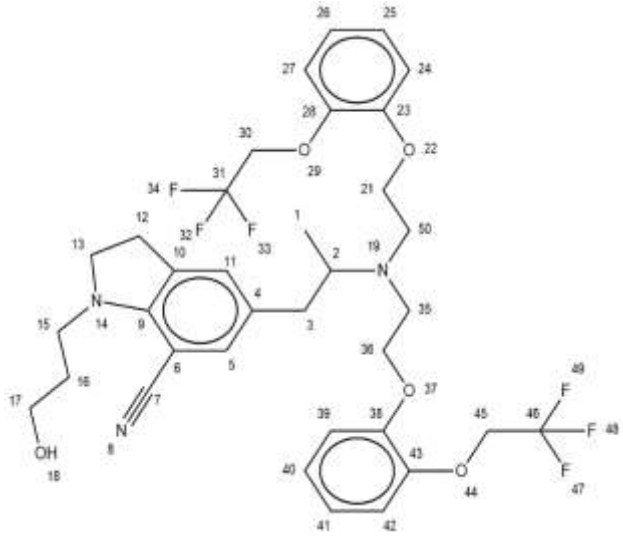

Fig. 7: Structure of Impurity 2.

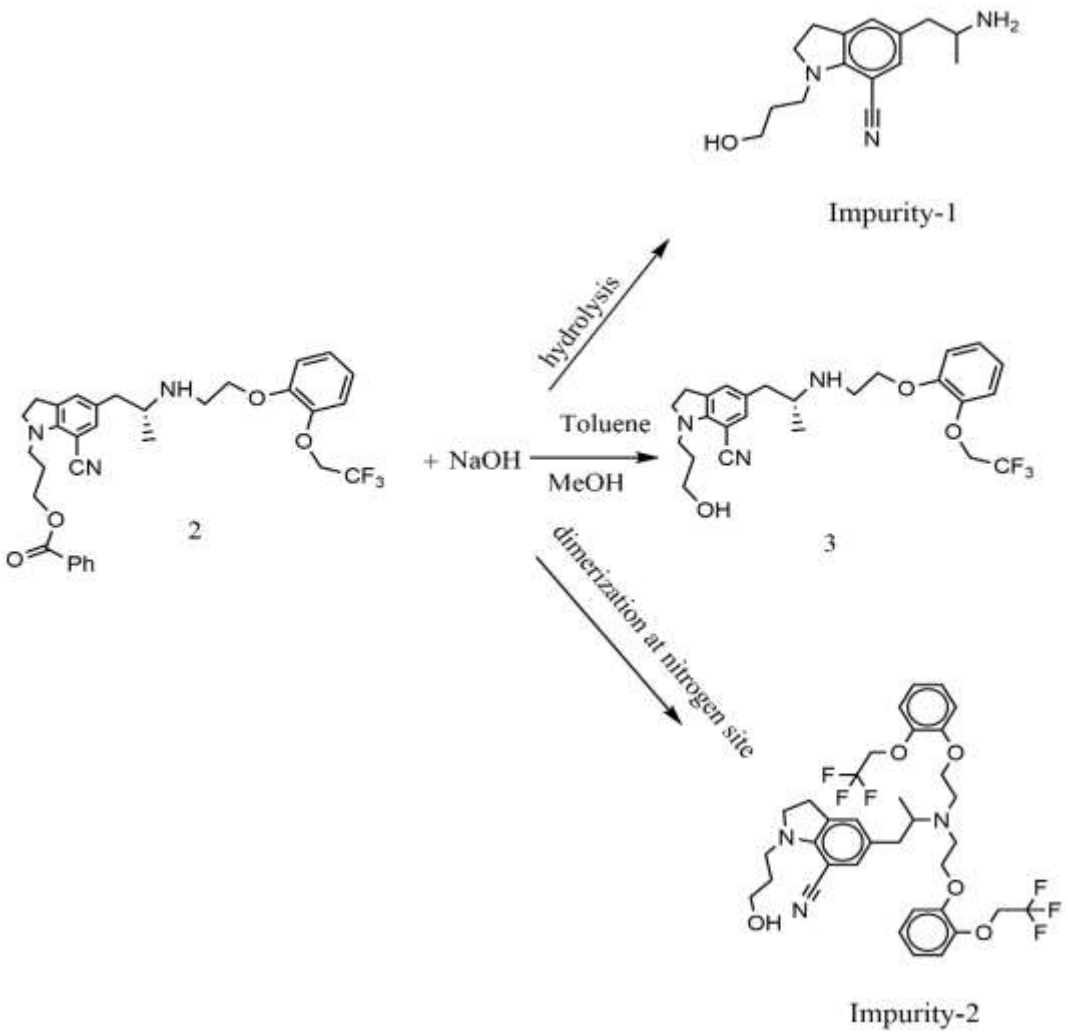

Fig. 8: Formation of Impurity 1 and Impurity 2 of Silodosin.

3.5. Analytical method validation

There is no method in the literature for detecting potential genotoxic impurities of silodosin. To quantify PGIs in silodosin at the TTC level, a method must be developed. The newly developed method is highly specific, selective and sensitive for quantification of PGI's in silodosin at the TTC level. The newly developed method was successfully validated as per ICH guidelines [14].

3.5.1. Specificity: The methods specificity was established by injecting individual impurity samples. Fig. 9 shows the HPLC spectrum of system suitability. Fig. 10 shows the silodosin spiked HPLC chromatogram with Impurity 1 and 2

at TTC level. No interference peaks in the diluent and silodosin samples were observed at the retention time of impurity 1 and impurity 2 indicating this method for determination of both the PGIs in silodosin showed good specificity.

Fig. 9: System Suitability.

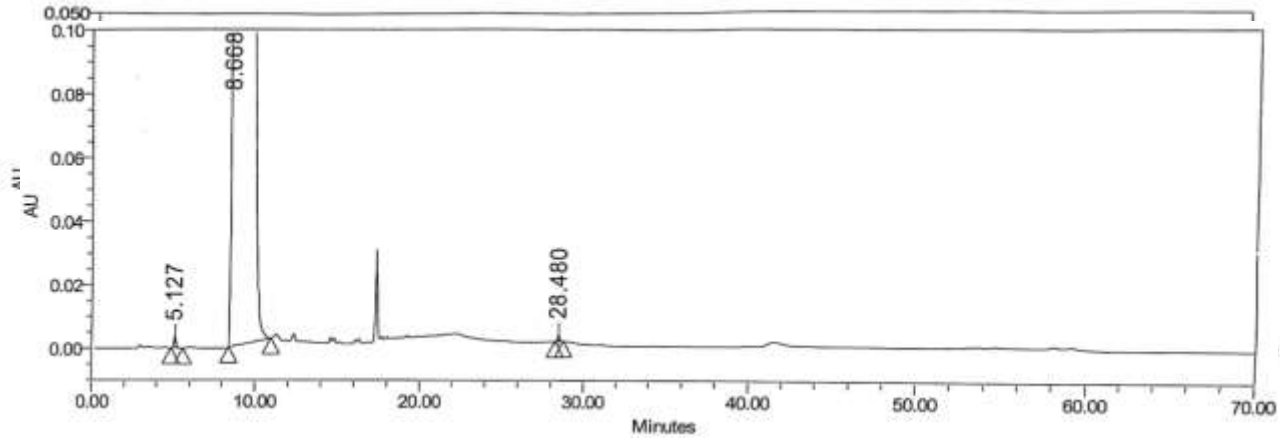

Fig. 10: Silodosin spiked chromatogram with Impurity 1 and 2 at TTC level.

3.5.2. Precision: The method's precision was confirmed using six replicate injections of a standard solution. Table 3 shows the precision study data of Impurity-2. The accuracy study data of Impurity-1is mentioned in Table 4. The % RSD for peak area of impurity 1 and impurity 2 was found to be 0.41% (Table 3) and 1.14% (Table 4), respectively.

Table 3: Precision study of Impurity-1

Entry	Name	Injections	Retention Time (min)	Area	Plate Count	Tailing
1	Impurity 1	1	5.365	32068	9825	1.10
2	Impurity 1	2	5.358	31958	9875	1.10
3	Impurity 1	3	5.332	31893	9788	1.10
4	Impurity 1	4	5.335	31796	9947	1.10
5	Impurity 1	5	5.321	31802	10093	1.09
6	Impurity 1	6	5.305	31700	10062	1.09
Mean			5.336	31870		
Std. Dev				131.4970		
% RSD				0.41		

Table 4: Precision study of Impurity-2

Entry	Name	Injections	Retention Time (min)	Area	Plate Count	Tailing
1	Impurity 2	1	28.793	16184	312144	1.06
2	Impurity 2	2	28.759	15972	314854	1.06
3	Impurity 2	3	28.697	15849	317445	1.05
4	Impurity 2	4	28.687	15709	317390	1.05
5	Impurity 2	5	28.623	15770	313717	1.03

6	Impurity 2	6	28.594	15740	312542	1.01
Mean			28.692	15871		
Std. Dev				180.1853		
% RSD				1.14		

3.5.3. Accuracy: The method's accuracy was determined by analysing samples spiked with the known concentration of both PGI's in silodosin sample at LOQ, 80%, 100% and 150% of the specified limit [15]. The impurity recoveries were calculated and are shown in Table 5 and Table 6.

Table 5: Accuracy study of Impurity-1

Level	**Solution**	**Concentration (in ppm)**	**Area obtained after spiking**	**% Recovery**	**Mean % Recovery**
LOQ	Test-1	0.0555	9351	97.29	95.85
	Test-2		9206	95.39	
	Test-3		9111	94.95	
80%	Test-1	0.1480	24920	96.27	96.73
	Test-2		24973	97.58	
	Test-3		24695	96.35	
100%	Test-1	0.1850	30872	96.18	96.18
	Test-2		31006	96.23	
	Test-3		31005	96.12	
150%	Test-1	0.2775	47183	97.12	97.15
	Test-2		46926	97.19	
	Test-3		46687	97.15	

Table 6: Accuracy study of Impurity-2

Level	**Solution**	**Concentration (in ppm)**	**Area obtained after spiking**	**% Recovery**	**Mean % Recovery**
LOQ	Test-1	0.0555	5008	104.62	103.67
	Test-2		5025	104.56	
	Test-3		4867	101.83	
80%	Test-1	0.1480	13064	101.34	101.55
	Test-2		12921	101.38	
	Test-3		13009	101.92	
100%	Test-1	0.1850	16179	101.21	100.60
	Test-2		16114	100.42	
	Test-3		16089	100.16	
150%	Test-1	0.2775	24520	101.35	101.98
	Test-2		24525	102.00	
	Test-3		24549	102.58	

3.5.4. LOD and LOQ: The lowest concentration that can be detected is LOD, whereas the lowest concentration that can be quantitatively quantified with reasonable accuracy is LOQ. The LOD and LOQ for impurity 1 and impurity 2 were found to be 0.0000018% and 0.0000055%.

3.5.5. Linearity: Six solutions were prepared containing 0.05, 0.09, 0.148, 0.185, 0.22 and 0.28 µg mL^{-1} impurities standard concentration which corresponded to 30, 50, 80, 100, 120 and 150%, respectively. Each solution was injected in triplicate. The result shows an excellent correlation between the peak area and concentration of both the PGIs which can be seen in the Table 7.

Table 7: Linearity study of Impurity-1 and Impurity-2

Impurity 1			**Impurity 2**		
Level	**Concentration (in ppm)**	**Mean Peak Area**	**Level**	**Concentration (in ppm)**	**Mean Peak Area**
LOQ	0.0555	10857	LOQ	0.0555	4876
50%	0.0925	18021	50%	0.0925	8188
80%	0.1480	28686	80%	0.1480	13240
100%	0,1850	35958	100%	0,1850	16174
120%	0.2220	43387	120%	0.2220	19993
150%	0.2775	54208	150%	0.2775	25149
Correlation coefficient		1.0000	0.9995		
slope		190429.87	89927.48		
Intercept		-81.39	-269.25		

3.5.6. Robustness: Experiment conditions such as mobile phase flow rate and the column oven temperature were deliberately changed to test the method's robustness. The mobile phase flow rate was adjusted at 0.7 mL min^{-1} and the same was altered to 0.6 to 0.8 mL min^{-1}. The influence of column oven temperature on the resolution was investigated at 32°C and 28°C (altered by 2°C). The results showed that changing the chromatographic conditions deliberately had no effect on the chromatographic performance for all impurities in the spiked sample, demonstrating the method's robustness if the mobile phase components were kept constant.

3.5.7. Solution stability: The stability of the solution was investigated by standard and test preparation. The solution was firmly enclosed in HPLC vial at 25°C for 35 hours and found stable with no significant change in the peak area.

4. CONCLUSION

Two unknown impurities were observed in the crude sample of silodosin with a concentration of more than 0.1%. According to ICH guidelines, these impurities must be identified, thus they are isolated using preparative HPLC and characterized using LCMS and NMR. The probable structure of the impurities is proposed, having structural alert as confirmed by Toxtree and VEGA results, which state that these impurities are potentially genotoxic. Impurity-1 forms as a result of uncontrolled hydrolysis while Impurity-2 forms due to dimerization at the nitrogen site. As these impurities are potentially genotoxic, they must be controlled at the TTC level for which an analytical method needs to be developed. A simple, sensitive and facile HPLC-UV method is developed and validated which will allow the determination and quantification of these impurities at a TTC level of 185 ppm. This study will facilitate to identification and control of these impurities in the final API, which in turn help to improve the safety and efficacy of the drug.

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DECLARATION OF CONFLICTING INTEREST

There are no conflicts of interest to declare.

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None

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