Toxicology International, *30(4) : 475-487; 2023. DOI: 10.18311/ti/2023/v30i4/34033*

Online ISSN : 0976-5131; Print ISSN : 0971-6580

Toxicology International

Contents available at: www.informaticsjournals.com/index.php/toxi



Optimization of Stability-Indicating HPLC Method for Analyzing Process Related Impurities of Penfluridol and Structural Elucidation of Stress Degradation Products by LCMS/MS

Beram Adilakshmi[1], V. K. Rohini[1], T. Eswarlal[2], Ch. Lakshmi Prasanna[3] and Venkateswara Rao Anna[1]*

[1]Department of Engineering Chemistry, College of Engineering, Koneru Lakshmaiah Education Foundation, Vaddeswaram, Guntur - 522302, Andhra Pradesh, India; avrchemistry@gmail.com

[2]Department of Engineering Mathematics, College of Engineering, Koneru Lakshmaiah Education Foundation, Vaddeswaram, Guntur - 522302, Andhra Pradesh, India

[3]Department of Engineering Chemistry, Sagi Rama Krishnam Raju Engineering College, Bhimavaram - 534204, Andhra Pradesh, India

Abstract

This study focused on the development of a simple and sensitive HPLC method for resolution and quantification of process-related impurities of penfluridol and further assessment of forced degradation behavior of penfluridol. The chromatographic separation was achieved on XTerra™ C18 (250×4.6 mm, 5.0µm) column and UV detection at 245nm. The mobile phase comprises of methanol and tetrahydrofuran in 55:45 (v/v) as solvent A and acetonitrile and tetrahydrofuran in 80:20 (v/v) as solvent B. The 60:40 (v/v) composition of solvent A and B were pumped isocratically at 1.0mL/min. In the proposed conditions, the retention time identified as 5.29 min for penfluridol, 4.51 min, 9.95 min and 7.64 min respectively for impurity 1, 2 and 3 with acceptable system suitability. The method produces sensitive detection limit of 0.008µg/mL for impurity 1, 2 and 0.004 µg/mL for impurity 3 with calibration range of 25-150 µg/mL for penfluridol and 0.025-0.150 µg/mL for impurities. The drug was exposed to different stressed conditions (acid, base, peroxide, thermal and UV light) according to ICH Q1A (R2) guidelines. The Degradation Products (DPs) formed during the stress study was characterized by LCMS/MS in ESI positive mode and the possible structures of five DPs with possible degradation pathways were proposed. The outcomes of other validation studies were likewise satisfactory and proven adequate for regular analysis of penfluridol and its process-related impurities in bulk drug and pharmaceutical dosage forms and can also applicable for evaluation of stress degradation mechanism of penfluridol.

Keywords: Characterization of Degradation Compounds, HPLC Method Development, Impurity Analysis, Penfluridol, Stress Studies

**Author for correspondence*

Article Received on: 16.06.2023 Revised on: 01.09.2023 Accepted on: 03.09.2023

1. Introduction

The pharmaceutical drug and drug product purity is of paramount importance to establish drug safety from the standpoint of patient exposure to degradation products and impurities[1]. The manufacturers are always implement suitable precautions to minimize the impurities and degradation products by selecting suitable synthesis route, excipient, process of formulation preparation and selection of appropriate packing[2]. Although, impurities are still likely to be encountered during the manufacturing process or degradation Active Pharmaceutical Ingredients (APIs) or due to the generation of leachable compounds from container closure system[3]. The regulatory agencies such as ICH (International Conference on Harmonization) issued guidelines to control the impurities in a pharmaceutical drug product and the USP (United States Pharmacopeia) included a chapter (16664) to provide guidance on leachable compounds testing and control[4,5].

It is important that highly selective and sensitive analytical methods are in place for the impurity investigations during the product development phase[6]. If any impurity is identified at a level representing toxicological risk, it should be adequately quantified and evaluated for safety assessments. Furthermore, based on the identification of the impurity and its source, attempts could be made to control or avoid its formation[7]. Currently, the development of efficient and economical analytical methods to cope with a large number of samples has become an important part in the pharmaceutical industry for producing safe pharmaceutical product.

Penfluridol is a highly potent, first generation diphenylbutylpiperidine class antipsychotic drug prescribed for the treatment of chronic schizophrenia and similar psychotic disorders[8]. It was considered as depot medication and was administered weekly once. It reduces the positive symptoms of schizophrenia and other psychotic disorders by blocking dopamine receptors particularly postsynaptic D2 receptor[9]. The side effects such as transitory acute dystonia, agranulocytosis, akathisias, dyskinesias, tremor, rigidity and gynaecomastia are less common during the usage of penfluridol[10].

In literature, one HPLC and MS/MS method reported for quantification of penfluridol in human plasma[11]. One HPLC method reported for quantification of penfluridol along with other antipsychotic drugs in plasma[12] and LCMS method reported for estimation of penfluridol in hair obtained from psychiatric patients along with other antipsychotic drugs[13]. Literature survey reveals no analytical method reported for the determination of penfluridol in pharmaceutical preparation. There is no official methods are included in major pharmacopeia's for the determination of process-related impurities in penfluridol formulations. There is no method reported for separation and identification of stress degradation compounds of penfluridol. Hence, in this study, novel stability-indicating reverse-phase HPLC methods was developed for the determination of process related impurities of penfluridol and further the stress Degradation Products (DPs) were characterized using LCMS analysis. Altogether, this new method would be of great value to ensure the safety and quality of penfluridol in synthetic material and formulations. The pure drug penfluridol was synthesized by Grignard reaction between *methyl 4-oxopiperidine-1-carboxylate* and *5-bromo-2-chlorobenzotrifluoride* followed by deprotection of urethane. Then the obtained product was alkylated with *1,1'-(4-Chlorobutylidene)bis(4-fluorobenzene)* produces penfluridol[14,15]. During the process of synthesis, reactants, intermediate products and the bi-products will left in the final product as impurity and were considered as process-related impurities. The molecular structure of penfluridol and its process-related impurities in the study were presented in Figure 1.

2. Materials and Methods

2.1 Chemicals and Reagents

The penfluridol analytical pure drug having 99.04% purity, its process-related impurities 1, 2 and 3 were procured from Mova Pharmaceutical Pvt Ltd, Chennai, Tamil Nadu. The pharmaceutical tablet formulation of penfluridol having brand Penridol® (20 mg) were purchased in local pharmacy. The solvent used in the study like methanol, acetonitrile were of HPLC grade and Milli-Q® water were purchased from Merck chemicals, Mumbai. The analytical reagent grade chemicals such as acetic acid, sodium acetate, Hydrochloric Acid (HCl), Sodium Hydroxide (NaOH), and hydrogen peroxide were purchased from Fisher scientific, Mumbai.

2.2 Equipment's

The HPLC study was conducted on Agilent 1100 (USA) equipment that was equipped with quaternary

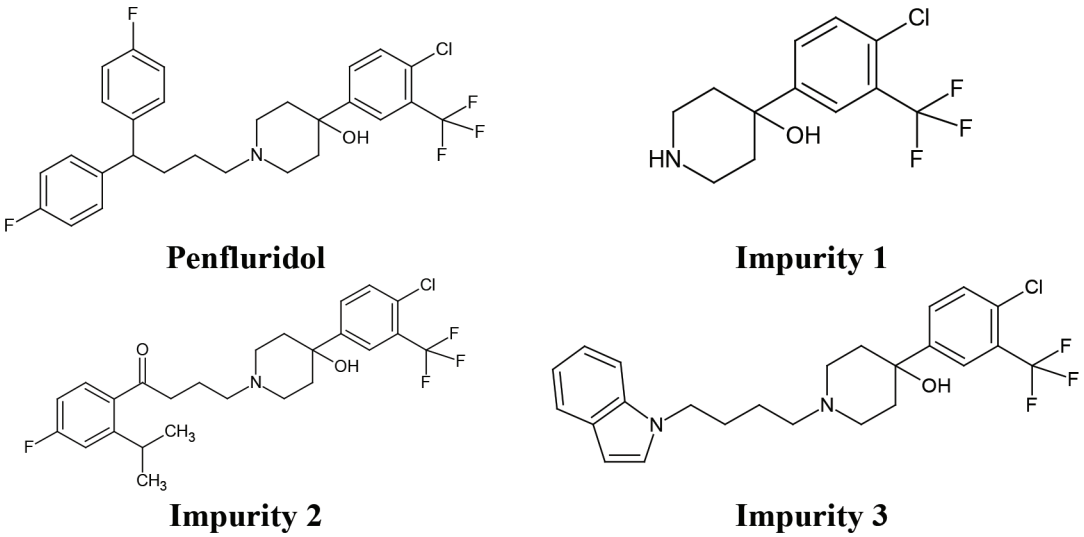


Figure 1. Structure of penfluridol and its process-related impurities in the study.

solvent delivery pump (model G1311 A). The prepared standard and samples were injected into the system using temperature temperature-adjustable auto sampler (model G 1329A) with 0.1–1500 µL injection capacity. The injected samples were resolved with suitable C18 column and the resolved samples were detected through programmable Ultraviolet (UV) detector (model G 1314 A). The detector response was integrated with Agilent chem-station version 2.0 software. The LCMS analysis was performed on Waters LCMS (Japan) equipped with triple quadruple mass detector and MassLynx software.

2.3 Preparation of Penfluridol and Impurity Solutions

The penfluridol standard drug at a quantity of 50 mg was weighed accurately in a sterilized volumetric flask half filled with diluent (methanol). The flask was kept in an ultrasonic bath sonicator for 2 min to dissolve penfluridol completely in diluent. The penfluridol dissolved solution was filtered through 0.2 µm nylon membrane filter in to a clean and dry 25 mL volumetric flask and the final volume made up to 25 mL using the same diluent. The penfluridol standard solution at 1 mg/mL (1000 µg/mL) was obtained and the same procedure was used for preparing impurity 1, 2 and 3 solutions in the same concentration level separately. Then the solutions were separately diluted to known fixed concentration and selected concentrations of standard and impurity solutions were mixed during the analysis.

2.4 Preparation of Formulation Solution

The Penridol® brand tablets of penfluridol with dosage of 20mg were used for preparing the formulation solution in the study. One complete strip of tablets was grinded with a sterile mortar and pestle to a uniform fine powder. The tablet powder equivalent to 25 mg penfluridol pure drug was weighed accurately into a sterile volumetric flask having 15 mL diluent. The penfluridol in the formulation powder was dissolved completely in the diluent with the help of an ultrasonic bath sonicator. The un-dissolved formulation excipients were removed by filtration through 0.2 µm nylon membrane filter and the volume was made up to the mark. The formulation solution at 1000 µg/mL concentration was obtained and was brought to precision level concentration in linearity before the analysis.

2.5 Method Development

The method development was initiated by identifying suitable wavelength for the detection of penfluridol and its impurities using UV detector. The UV-visible spectrophotometer was used for the identification of suitable wavelength for the detection of penfluridol and its impurities. The standard solution at 10µg/mL concentration of penfluridol and its impurities was scanned individually at a scan range of 400-200 nm. The overlay UV absorption spectra of penfluridol and its impurities confirm the iso-absorption wavelength that was suitable for the detection of penfluridol and its impurities.

The stationary phase selection was carried by analysing the standard concentration of penfluridol spiked with 0.1% impurities solution on different columns. During the column selection, different columns with different configuration were studied for best resolution of analytes. The composition, pH and flow of mobile phase were optimized by change in different solvent composition with different pH and flow rates. In each method development trail condition, precision level concentration of penfluridol spiked with 0.1% impurities solution was analysed. The chromatographic response, peak shape and system suitability were summarized and the conditions that produce best results were considered as suitable for the analysis penfluridol and its impurities.

2.6 Method Validation

The method developed in the study was validated for its acceptable performance to ensure suitability of indent purpose. The parameters such as range, linearity, accuracy, precision, specificity, detection limit, quantification limit, ruggedness and robustness experiments were executed as per reported literature[16-23] and ICH guidelines[24,25].

The Limit Of Quantification (LOQ) and Limit Of Detection (LOD) for impurities were confirmed by measuring response at signal to noise ratio of 10:1 and 3:1, respectively, by injecting progressively known concentrations of impurity solutions in the optimized HPLC method. The precision experiment was also performed at LOQ level by injecting six independent preparations of impurities in the same day (n = 6) for intraday precision and three different days (n = 2 in each day) for interday precision. The % RSD (relative standard deviation) of the peak areas observed for each impurity was determined.

Linearity was evaluated by analysing the mixed calibration standard solutions at six concentration levels. The linearity solutions were prepared from LOQ level and regression equations were plotted with the least squares linear regression method. The method precision was investigated by analysing six individual preparations in the same day for intraday precision, three different days by same analyst for interday precision and six times by six different analysts in the same day in ruggedness. The method robustness was verified by introducing minor variations in the developed method parameters, including the mobile phase mixture, pH, and detector wavelength and column temperature. The selectivity, resolution between each nearby peak and the % change in the peak area response were evaluated in each of the altered condition. The method accuracy was evaluated by spiking pre-analysed samples with four different concentration levels in triplicate, i.e., at LOQ, 50%, 100% and 150% levels of the specification limit. The % recovery in each analysis and the % RSD of recovery in each spiked level was summarized to evaluate the method accuracy.

The specificity and stability indicating power of the developed method was evaluated by performing stress degradation study. Various stress studies like acid, base, peroxide, thermal and ultraviolet stress studies were performed for standard penfluridol. The placebo, penfluridol pure drug and tablet powder were exposed 5 mL of hydrochloric acid (1 N), 5 mL of sodium hydroxide (1 N) and 5 mL of hydrogen peroxide (3.0%) separately for acid, base and peroxide degradation study respectively for 12 hours. Then, the stress-exposed samples were neutralized with suitable diluent. The samples were kept in a photolytic chamber irradiated with UV light at 1.2 million Lux/h and 1,03,959 Wh/m^2 for UV light degradation study and samples were kept in an air oven at 80°C for 24 hours for thermal degradation study. The all the stress exposed samples were diluted to target concentration and then the diluted samples were analysed in the developed method. The resultant chromatograms and chromatographic response were utilized for evaluating the stability indicating nature of the proposed method.

The stress degradation compounds (DPs) were characterized using mass spectral analysis. The mass spectral analysis was performed by allowing 40% of the column eluents into the column using a splitter and the samples were recorded using mass detector that was operated in ESI (Electrospray Ionization) positive ion mode. The mass detector was operated with adequate conditions such as capillary voltage (3700 V), fragment voltage (65 V) and skimmer voltage (70 V). Nitrogen gas was utilized for drying (350°C, 7 L/h) and nebulization (45 Psi). The spectra throughout the analysis were recorded under similar experimental conditions and 20-30 average scans were conducted.

The proposed method was applied for identification and quantification of process-related impurities of penfluridol in formulations. The Penridol® formulation solution spiked with known concentration of impurities and the un-spiked solution was analysed in the proposed method. The % assay of impurities was calculated by comparing peak area response of each impurity in its corresponding calibration curve.

3. Results and Discussions

As the literature doesn't show any analytical method for quantification of process related impurities of penfluridol, this study aimed to develop a simple HPLC method for identification and quantification of process related impurities 1, 2 and 3 of penfluridol. Different configurations of columns including Octadecylsilane (ODS), phenyl–hexyl, cyano and amino columns were studied for best resolution of analytes. The mobile phase composition with suitable pH range was optimized by varying different solvent compositions including various strengths of buffers. The hydrophilic ionisable functional groups present in analytes such as –C=O, –OH etc., were effectively resolved with mobile phase containing pH buffers and hence various buffers with different pH range was studied for effective resolution. In the use of phosphate buffer in the mobile phase produces asymmetric peaks with poor resolution was noticed. The presence of methanol in the mobile phase doesn't resolve the analytes. Hence these were not studied in the optimization process. The mobile phase with low pH value shows negative influence on the column life and hence mobile phase was selected such that the pH doesn't influence the column life. The optimized separation of penfluridol and its impurities was achieved on XTerra™ C18 (250 × 4.6 mm, 5.0 µm) column and UV detection at 24 5nm. The mobile phase solvents was prepared by mixing methanol and tetrahydrofuran in the ratio of 55:45 (v/v) as solvent A and acetonitrile and tetrahydrofuran in 80:20 (v/v) as solvent B. The 60:40 (v/v) composition of solvent A and B were pumped isocratically at 1.0 mL/min. The column oven temperature (35°C) and sample volume (20µL) was kept constant throughout the analysis.

In the developed conditions, the shape of the peaks corresponds to penfluridol and its impurities were noticed to be symmetric and the resolution between the nearby peaks was observed to be more than 2. The compounds in the standard solution were identified by injecting the individual standard solution and comparing the individual retention time with standard. The retention time of the analytes was noticed to be 5.29 min for penfluridol, 4.51 min for impurity 1, 9.95 min for impurity 2 and 7.64 min for impurity 3. The column efficiency for penfluridol and its all impurities was noticed to be lesser than the limit in case of tail factor and greater than acceptable limit in case of theoretical plates and resolution and results were illustrated in Table 1. Placebo and the standard penfluridol solution spiked with impurities chromatogram were presented in Figure 2 and based on chromatograms it was confirmed that the method was specific for the separation and detection of process related impurities of penfluridol.

System suitability data indicated that the system was suitable for use as the tailing factor for all the analytes was less than 1.5 and the resolution between any of the two adjacently eluting analytes was greater than 2.5. It also confirms the good selectivity of the method. The s/n method was utilized for evaluating method sensitivity and results were expressed in terms of LOD and LOQ. The detection limit was observed to be 0.008 µg/mL for impurity 1, 2 and 0.004 µg/mL for impurity 3. The results obtained for impurities indicated the higher sensitivity of the method.

The higher quantification limit concentration of impurities i.e., 0.025 µg/mL was taken as initial concentration for constructing the calibration curve for impurities. The penfluridol standard solution was prepared such that the

Table 1. Results observed in method validation

Parameter	Results			
	Penfluridol	Impurity 1	Impurity 2	Impurity 3
System suitability[$]				
t_R (min)	5.29	4.51	9.95	7.64
RRT	--	0.85	1.88	1.44
RRF	--	0.049	0.038	0.062
R_S	3.56	--	7.46	8.91
A_S	1.03	1.08	0.92	1.06
N	5418	6207	7141	8959
Linearity				
Range in µg/mL	25-150	0.025-0.150	0.025-0.150	0.025-0.150

Table 1. *Continued....*

Parameter	Results			
	Penfluridol	**Impurity 1**	**Impurity 2**	**Impurity 3**
Slope	8444.4	449689	358705	520276
Intercept	38497	– 1778.7	– 2467	1994.5
r^2	0.9991	0.9998	0.9995	0.9996
Precision[$$]				
Intraday	0.19	0.68	0.26	0.85
Interday (day 1)	0.16	0.90	0.54	0.90
Interday (day 2)	0.22	0.61	0.80	0.30
LOQ level	--			
Accuracy at 50 % level[$]				
Amount added (µg/mL)	50	0.050	0.050	0.050
Recovered (µg/mL)	49.393	0.050	0.049	0.050
% Recovery	98.79	99.06	98.70	99.06
% RSD	0.77	0.66	0.95	0.64
Accuracy at 100 % level[$]				
Amount added (µg/mL)	100	0.10	0.10	0.10
Recovered (µg/mL)	99.107	0.099	0.099	0.099
% Recovery	99.11	98.92	99.19	98.98
% RSD	0.71	0.87	0.62	0.74
Accuracy at 150 % level[$]				
Amount added (µg/mL)	150	0.150	0.150	0.150
Recovered (µg/mL)	148.815	0.149	0.149	0.151
% Recovery	99.21	99.07	99.01	100.51
% RSD	0.85	0.57	0.68	0.58
Sensitivity				
LOD (µg/mL)	--	0.008	0.008	0.004
LOQ (µg/mL)	--	0.025	0.025	0.013

t_R (min) = retention time; RRT = relative retention time; RRF = relative response factor; R_S = resolution; K^1 = retention factor; A_S = tail factor; N = No. of theoretical plates; r^2 = slope; [$]average of three determinations; [$$]average of six determinations.

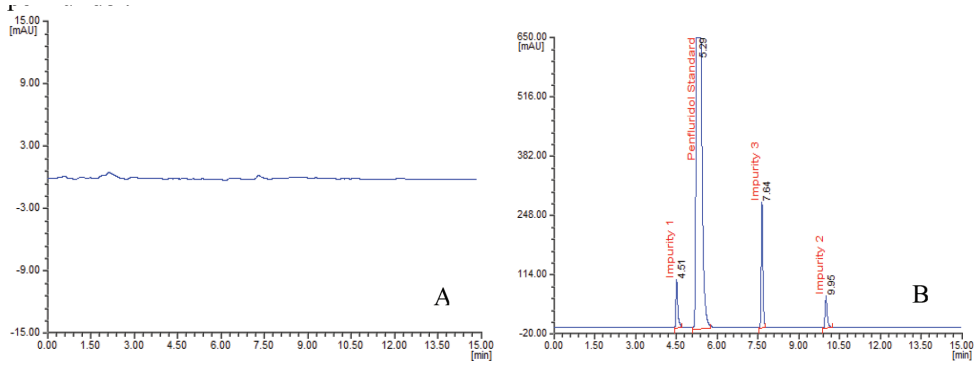


Figure 2. Chromatograms observed in specificity study. Placebo analysis chromatogram (A) and chromatogram observed for analysing penfluridol standard solution spiked with 0.1 % impurities.

solution contains 0.1% of each impurity and an accurate fit calibration curve was obtained in the concentration level of 25-150 µg/mL for penfluridol and 0.025-0.150 µg/mL for impurities in the study. The calibration curve was found to be linear with very high correlation coefficient for both impurities and standard penfluridol.

The %RSD values of peak areas obtained were less than 2 for impurities and penfluridol in intraday, interday precision, precision at LOQ level and ruggedness study indicating good precision of the method. Accuracy of the method was determined spiked recovery studies by spiking 50%, 100% and 150% concentrations for penfluridol spiked with 0.1µg/mL for impurity 1, 2 and 3. The % recovery was calculated for standard and all impurities in each analysis and the % RSD was calculated for each spiked level. The acceptable % recovery of 98-102 was observed for penfluridol and impurities studied and the % RSD in each spiked level was found to be less than 2 which in the acceptable limit confirm the accuracy of the method. The summary results observed in system suitability, linearity, precision, accuracy and sensitivity study in the proposed method were presented in Table 1.

There is no significant change in the chromatographic response and system suitability was noticed when the experiment was conducted with minor variations in the proposed method conditions. The resolutions between any two adjacent analytes were obtained greater than 2.0 and tailing factors of all analytes were under acceptable. The variability in the estimation of penfluridol and impurities was within the acceptable level of less than 2 indicating the robustness of the method. Table 2 presents the robustness study results obtained in the developed method.

Table 2. Robustness results in the developed method

S. No.	Changed condition	Parameter	Results observed			
			Penfluridol	Impurity 1	Impurity 2	Impurity 3
1	MP 1	% change	0.61	0.14	0.60	0.42
		t_R	5.04	4.52	9.97	7.66
		N	6068	6238	7170	8995
2	MP 2	% change	0.29	0.92	0.34	0.83
		t_R	5.02	4.53	9.99	7.67
		N	6014	6223	7159	8981
3	pH 1	% change	0.58	-0.37	0.80	0.60
		t_R	5.07	4.54	9.94	7.63
		N	6155	6244	7130	8946
4	pH 2	% change	0.53	-0.20	0.72	0.11
		t_R	4.97	4.53	10.00	7.68
		N	6003	6238	7177	9004
5	WL 1	% change	0.67	0.48	0.39	0.48
		t_R	5.10	4.54	9.92	7.61
		N	5483	6248	7116	8928
6	WL 2	% change	0.86	0.11	0.17	0.83
		t_R	5.02	4.55	9.94	7.63
		N	5429	6260	7133	8949
7	CT 1	% change	0.81	0.48	0.77	0.42
		t_R	5.29	4.50	9.98	7.66
		N	5906	6198	7162	8986
8	CT 2	% change	0.40	0.26	0.62	0.30
		t_R	5.34	4.50	9.94	7.63
		N	5998	6192	7130	8946

[$]Average of three determinations.

Forced degradation studies were conducted to evaluate the effectiveness of the method for resolution of degradation compounds and the study was conducted in acid, base, peroxide, thermal and UV light degradation conditions. There is no considerable degradation was noticed in thermal and peroxide degradation conditions with % degradation of 98.47% and 99.32% respectively. Among the degradation conditions, high % degradation was noticed in base degradation study with a % degradation of 8.59%. The chromatogram observed in this study (Figure 3A) show well resolved DPs at t_R of 6.13min and 8.24min respectively and were designated as DP 3 and 4 respectively. The chromatogram identified in acid degradation study (Figure 4B) clearly resolve two

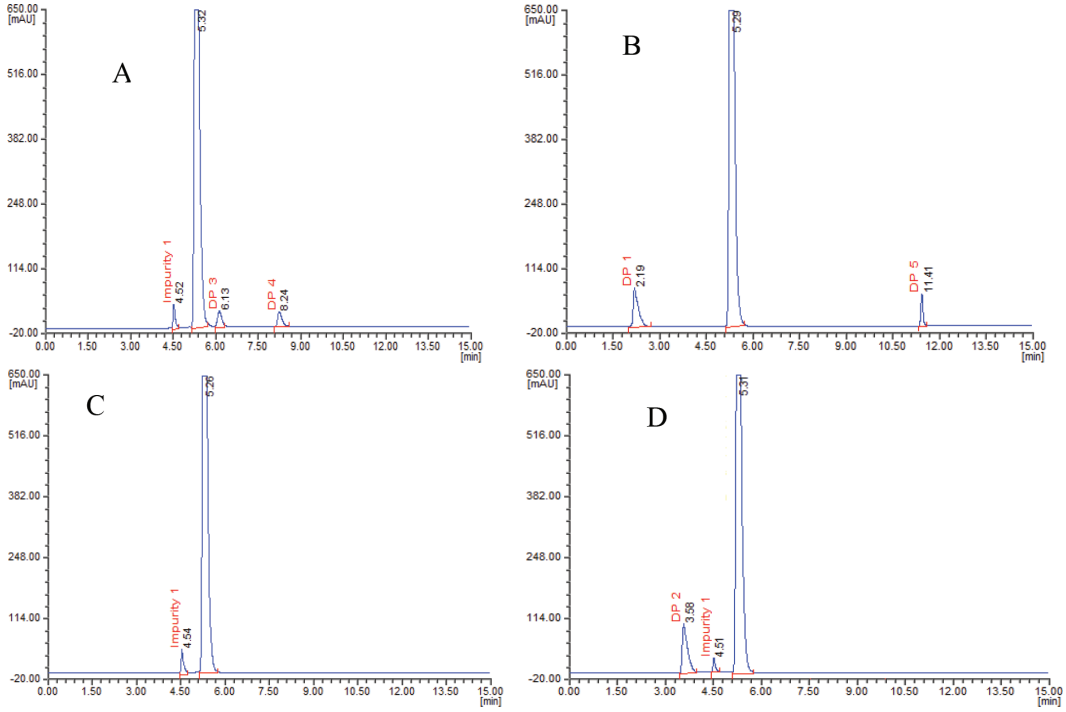


Figure 3. Forced degradation chromatograms of penfluridol. **(A)** Base degradation chromatogram of penfluridol showing DP 3 and 4; **(B)** Acid degradation chromatogram of penfluridol showing DP 1 and 5; **(C)** Peroxide degradation chromatogram of penfluridol; **(D)** UV light degradation chromatogram of penfluridol showing DP 2.

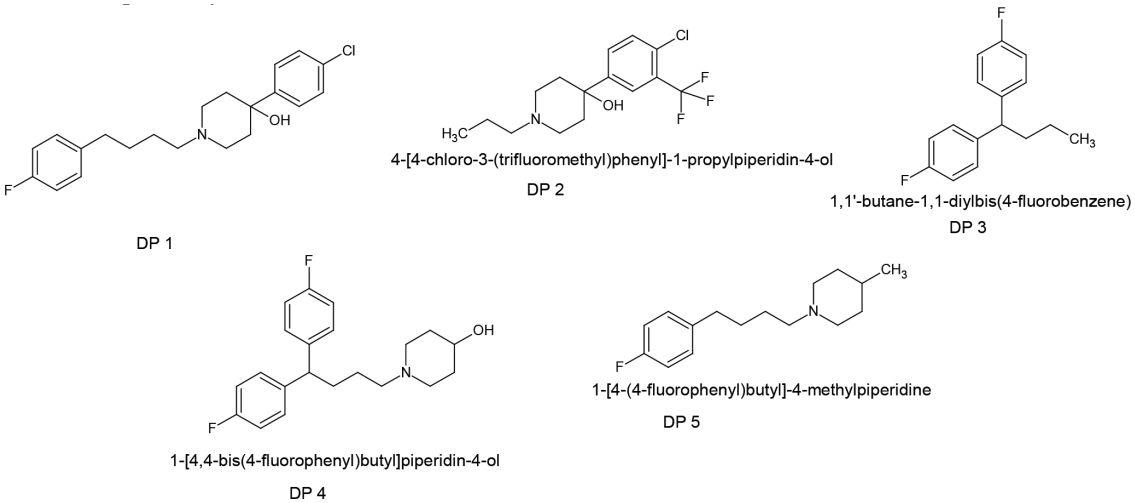


Figure 4. Molecular structure of DPs formed during forced degradation study of penfluridol. Mass spectra identified at t_R of 2.1 min for DP 1 **(A),** 3.5 min for DP 2 **(B),** 6.1 min for DP 3 **(C)**, 8.2 min for DP 4 **(D)** and 11.4 min for DP 5.

degradation products at t_R of 2.19min and 11.41min and were designated as DP 1 and DP 5 respectively with a % degradation of 7.92 %. The % degradation of penfluridol in UV light degradation was calculated to be 6.643 % with mass balance of 99.06 % and the chromatogram observed in this study clearly resolve one DP at t_R of 3.58 min (DP 2). The peak corresponding to impurity 1 at t_R of 4.5 min was noticed in the chromatograms of base, peroxide and UV light degradation chromatogram. Peak purity test results obtained from the PDA detector confirmed that the penfluridol peak was pure and homogeneous in all the analyzed stress samples. The mass balance of stressed samples was found to be more than 98%. Peak purity test results obtained confirmed that the penfluridol peak was homogeneous and pure in all the analysed stress samples. Insignificant change in assay of penfluridol in the presence of impurities and peak purity results of stress samples confirm the specificity and stability-indicating ability of the developed method. Table 3 presents the results and Figure 3 shows the representative chromatograms observed in forced degradation study.

Table 3. Results achieved during the forced degradation study of penfluridol

Stress condition	% degradation[$] of penfluridol	% assay[$] of penfluridol	% Mass balance[$] (assay + total impurities	Remark
Acid	7.92	92.08	98.58	DP 1 and 5 were identified
Base	8.59	91.41	98.19	DP 3 and 4 were identified
Peroxide	5.25	94.75	99.32	No DPs identified
Thermal	3.21	96.79	98.47	No DPs identified
UV light	6.43	93.57	99.06	DP 2 wad identified

[$]Average of three replicate experiments.

3.1 Characterization of DPs by LCMS/MS

The results observed in the degradation study proved that five DPs (DP 1 to DP 5) of penfluridol were formed and were well resolved using the proposed method. The DPs identified were characterised using LCMS/MS analysis. The mass spectral response identified for the DPs suggest that all the DPs exhibited abundant protonated molecular ions ($[M+H]^+$) in positive ionization mode. The collision-induced dissociation spectra of the molecular ions of penfluridol and its DPs were recorded to obtain structural information.

The ESI MS spectrum of DP 1 (Figure 5A) identified at t_R of 2.1 min show abundant parent ion at m/z of 362 (m+1) with most possible molecular formula of $C_{21}H_{25}ClFNO$. In addition, the spectrum also showed low abundant product ions at m/z of 278 (m+1) and 97 (m+1) with molecular formula of $C_{19}H_{16}FN$ and C_5H_5NO respectively. Based on the fragmentation pattern, the compound was identified as *4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)butyl]piperidin-4-ol* with molecular formula of $C_{21}H_{25}ClFNO$ and 362 g/mol as molecular mass. The proposed mass fragmentation pattern of DP 1 was presented in Figure 6 and its molecular structure was presented in Figure 4.

The mass fragmentation spectra of DP 2 (Figure 5B) show an abundant parent ion at m/z of 322 (m+1) under positive ionization mode. The spectrum also shows fragment ion at m/z of product ions at m/z of 247 (m+1) by losing C_3H_9NO. The spectrum shows abundant fragment ion at m/z of 97 (m+1) corresponds to pyridin-4-yloxonium ion with molecular formula of C_5H_5NO. The elemental compositions of molecular ion of DP-2 and all its fragmentation ions have been confirmed by accurate mass measurements. Based on the achieved date, the DP 2 was confirmed as 4-[4-chloro-3-(trifluoromethyl) phenyl]-1-propylpiperidin-4-ol with molecular formula of $C_{15}H_{19}ClF_3NO$. The proposed mass fragmentation pattern of DP 2 was presented in Figure 6 and its proposed molecular structure was presented in Figure 4.

The ESI-MS spectrum of DP 3 (Figure 5C) identified at retention time of 6.1 min show parent ion at m/z of 247 (m+1) with abundant fragment ion at m/z of 110 (m+1).

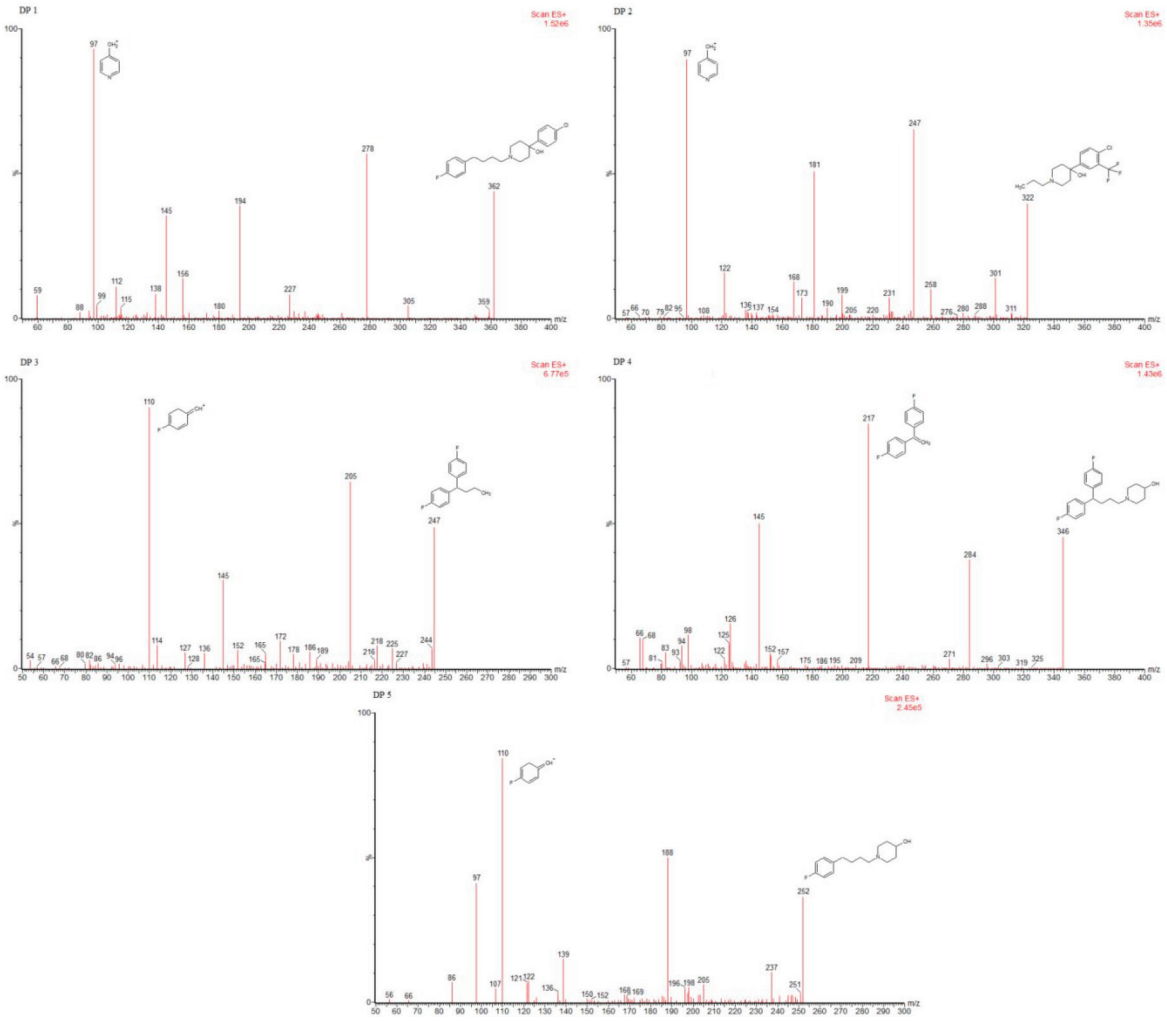


Figure 5. Mass fragmentation spectra of DPs formed during forced degradation study of penfluridol.

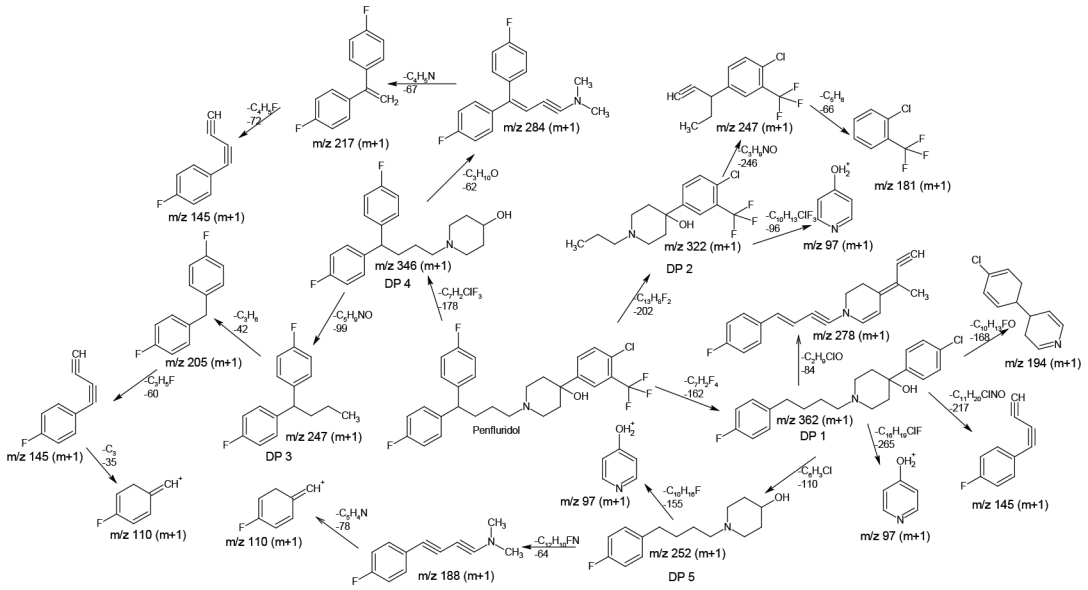


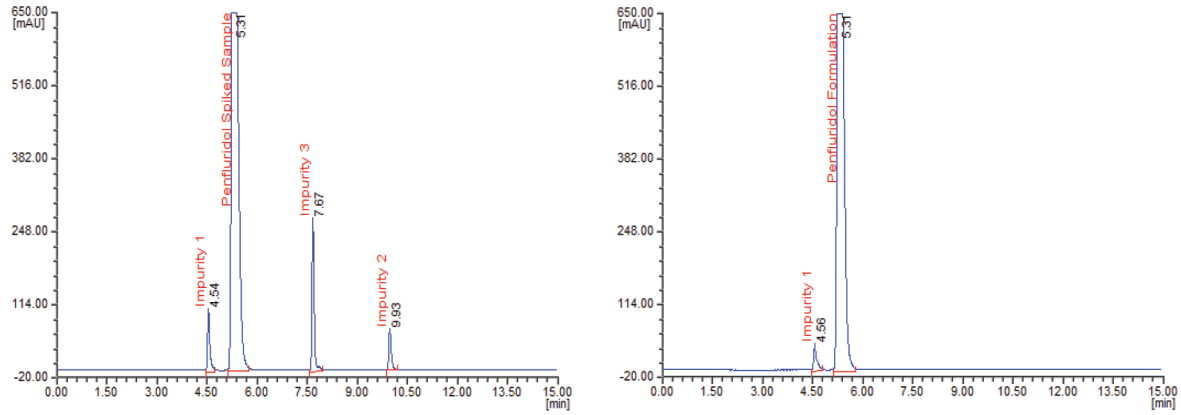
Figure 6. Mass fragmentation pattern of DPs of penfluridol.

The parent ion shows molecular formula of $C_{16}H_{16}F_2$ that show the fragment ion with molecular formula of 205 (by losing C_3H_6) and 110 (by losing C_6H_5F). The elemental compositions of molecular ion of DP 3 and all its fragmentation ions have been confirmed by accurate mass measurements. The peak purity studies of DP 3 confirm it as one of the degradation products of DP 4 observed in the study. The structure of the DP was shown in Figure 4 and its mass fragmentation mechanism was presented in Figure 6.

The ESI-MS spectrum identified at a retention time of 8.2 min (Figure 5D) show parent ion at m/z of 346 corresponding to the $[M+H]^+$ of DP 4 formed in the study. The spectrum showed abundant product ions at m/z 217 (m+1). The peak purity test and Collision-Induced Dissociation (CID) studies of DP 4 confirms its molecular structure. All these product ions and patent ion confirms DP 4 as *1-[4,4-bis(4-fluorophenyl)butyl]piperidin-4-ol* with molecular formula $C_{21}H_{25}F_2NO$. The structure of the DP 5 was shown in Figure 4 and its mass fragmentation mechanism was presented in Figure 6.

The ESI MS spectrum $[M+ H]^+$ ion of the DP 5 (Figure 5E) showed abundant product ions at m/z 252 (m+1) with abundant product ion at m/z 110. The peak purity CID studies of DP 5 confirm it as one of the degradation products of DP 1 observed in the study. The elemental compositions of the molecular ion of DP 5 and all its fragmentation ions have been confirmed by accurate mass measurements. Based on the studies, the DP 5 was identified as *1-[4-(4-fluorophenyl)butyl] piperidin-4-ol* with a molecular mass of 252g/mol and a formula of C15H22FNO. Its molecular structure and fragmentation mechanism was presented in Figures 4 and 6 respectively.

The developed HPLC method was applied for the quantification of process-related impurities of penfluridol in pharmaceutical formulation. The formulation sample directly analysed for the evaluation of impurities present in it and also the formulation sample spiked with impurities was analyzed to evaluate the effectiveness of the method for the resolution and quantification of impurities in the formulation. The chromatogram obtained for impurities spiked formulation solution (Figure 7) show clear identification of peaks corresponds to impurities in the study. Whereas the chromatogram observed for un-spiked formulation solution show only one peak corresponds to impurity 1 in the study. This suggests that only impurity was identified in the sample whereas the quantity of other impurities in the sample was less than the detection limit. This confirms that the quantity of impurity in the sample was noticed to be under the permissible level and the proposed method can successfully applicable for the quantification of process-related impurities in penfluridol.



Spiked formulation chromatogram

Un-spiked formulation chromatogram

Figure 7. Formulation chromatogram of penfluridol in the developed method.

4. Conclusion

Penfluridol was subjected to forced degradation studies (acid, base, peroxide, thermal and UV light) as per ICH prescribed conditions. The drug penfluridol was found to be extremely stable in thermal and UV light degradation conditions and found susceptible to acidic, base and peroxide degradation. There was no significant degradation observed in thermal and UV light degradation conditions. A total five DPs were formed

under various vulnerable stress conditions and were characterized using LCMS/MS analysis. All the five DPs were successfully characterized in ESI-positive mode. The most probable mechanisms and pathways of all the DPs of penfluridol to the characterized products were outlined and discussed. The current study may be very useful for identifying process-related impurities and other possible DPs that may be there in bulk drugs at trace levels. This analytical method can be useful for the estimation of quality control samples for regular and stability studies of penfluridol and its process-related impurities.

5. Acknowledgements

The authors wish to acknowledge Department of Engineering Chemistry, Koneru Lakshmaiah Education Foundation for providing necessary facilities to carry out this work.

6. References

1. Holm R, David PE. Analytical advances in pharmaceutical impurity profiling. Eur J Pharm Sci. 2016; 87:118-35. https://doi.org/10.1016/j.ejps.2015.12.007
2. Paskiet D, Jenke D, Ball D, Houston C, Norwood DL, Markovic I. The Product Quality Research Institute (PQRI) leachables and extractables working group initiatives for Parenteral and Ophthalmic Drug Product (PODP), PDA. J Pharm Sci Technol. 2013; 67(5):430-47. https://doi.org/10.5731/pdajpst.2013.00936
3. International Conference on Harmonization B. Q3B (R2): Impurities in New Drug Products. Guidel: ICH Harmon. Tripart; 2006. p. 12. https://doi.org/10.1017/CBO9781107415324.004
4. Olsen BA, Sreedhara A, Baertschi SW. Impurity investigations by phases of drug and product development. TrAC Trends Anal Chem. 2017; 101:17-23. https://doi.org/10.1016/j.trac.2017.10.025
5. Mark HK, David E, Andrew T, Mark DM, Alan PM, Steven WB. Strategies to address mutagenic impurities derived from degradation in drug substances and drug products. Org Process Res Dev. 2015; 19(11):1447-57. https://doi.org/10.1021/acs.oprd.5b00091
6. Wei Y, Wu Y, Zhu T, Li Z, Zhang Y. Identification of UV-absorbing extractables from rubber closures used in containers of injectable powder and safety assessment of leachables in the drug. J Pharm Biomed Anal. 2017; 138:256-66. https://doi.org/10.1016/j.jpba.2017.02.023
7. Jacobson-Kram D, McGovern T. Toxicological overview of impurities in pharmaceutical products. Adv Drug Deliv Rev. 2007; 59(1):38-42. https://doi.org/10.1016/j.addr.2006.10.007
8. Van Praag HM, Schut T, Dols L, van Schilfgaarden R. Controlled trial of penfluridol in acute psychosis. Br Med J. 1971; 18(4):710-3. https://doi.org/10.1136/bmj.4.5789.710
9. Tuan NM, Lee CH. Penfluridol as a candidate of drug repurposing for anticancer agent. Molecules. 2019; 24(20):3659. https://doi.org/10.3390/molecules24203659
10. Zoe Clarke, Penfluridol, editor(s): Enna SJ, Bylund DB. xPharm: The comprehensive pharmacology reference. Elsevier. 2007; 1-4. https://doi.org/10.1016/B978-008055232-3.62384-0
11. Walia A, Rajput M, Lahoti N, Bhosale V, Khan A, Yadav R, Patil S. Analysis of anti-psychotic drug penfluridol in human plasma by HPLC and MS/MS. Trends Drug Deliv. 2022; 9(2):36-45.
12. Garay GL, Forfar-Bares I, Pehourcq F, Jarry C. Simultaneous determination of four antipsychotic drugs in plasma by high-performance liquid chromatography: Application to management of acute intoxications. J Chromatogr B. 2003; 795(2):257-64. https://doi.org/10.1016/S1570-0232(03)00576-2
13. Weinmann W, Muller C, Vogt S, Frei A. LC-MS-MS analysis of the neuroleptics clozapine, flupentixol, haloperidol, penfluridol, thioridazine, and zuclopenthixol in hair obtained from psychiatric patients. J Anal Toxicol. 2002; 26(5):303-7. https://doi.org/10.1093/jat/26.5.303
14. Sindelar K, Rajsner M, Cervena I, Valenta V, Jílek JO, Kakac K, Holubek J, Svatek E, Miksik F, Protiva M. Neurotropic and psychotropic agents. LXVII. 1-[4,4-Bis(4-fluorophenyl) butyl]-4-hydroxy-4-(3-trifluoromethyl-4-chlorophenyl) piperidine and related compounds: New synthetic approaches. Collect Czech Chem Commun. 1973; 38(12):3879-901. https://doi.org/10.1135/cccc19733879
15. Miroslav R, Antonin D, Miroslav P. Esters of 1-[4,4-bis(4-fluorophenyl)butyl]-4-(4-chloro-3-trifluoromethylphenyl)-4-piperidinol. Collection of Czechoslovak Chemical Communications. 1981; 46(6):1530-3. https://doi.org/10.1135/cccc19811530
16. Bikshal BK, Useni RM, Venkateswara RA, Maheshwara RL. Intended high-performance liquid chromatography procedure for the quantification of norfloxacin and its potential impurities in active pharmaceutical ingredient and tablet dosage forms. Thai J Pharm Sci. 2018; 42(1): 27-36.
17. Mallu UR, Anna VR, Kasimala BB. Rapid stability indicating HPLC method for the analysis of leflunomide and its related impurities in bulk drug and formulations.

Turk J Pharm Sci. 2019; 16:457-65. https://doi.org/10.4274/tjps.galenos.2018.34635

18. Kasimala BB, Anna VR, Mallu UR. Stability-indicating reversed-phase HPLC method for the separation and estimation of related impurities of cilnidipine in pharmaceutical formulations. Indian Drugs. 2019; 55(12): 41-9. https://doi.org/10.53879/id.55.12.11185
19. Sri Girija K, Kasimala BB, Anna VR. A new high-performance liquid chromatography method for the separation and simultaneous quantification of eptifibatide and its impurities in pharmaceutical injection formulation. Int J App Pharm. 2021; 13(2):165-72. https://doi.org/10.22159/ijap.2021v13i2.39895
20. Varma RB, Rao BS. Gas chromatography-head space-flame ionization sensor-based assessment of four residuary solvents in rivaroxaban bulk medication. Research Journal of Pharmacy and Technology. 2022; 15(11):5158-63. https://doi.org/10.52711/0974-360X.2022.00868
21. Varma Rajesh BH, Sreenivasa Rao B. Gas Chromatography-head space-mass spectrometry sensor based quality control of dobutamine hydrochloride bulk material for a mutagenic impurity, 2-bromopropane. Research Journal of Chemistry and Environment. 2023; 27(2):54-61. https://doi.org/10.25303/2702rjce054061
22. Bhupatiraju RV, Rao BS, Kapavarapu MVNR, Reddy MV. A novel Rivaroxaban degradation impurity detection by RP-HPLC extraction by preparative chromatography, and characterization by LC-MS, NMR and FT-IR: Analysis of novel impurity in batch samples and tablets of Rivaroxaban. Rasayan Journal of Chemistry. 2022; 15(4):2373-81. https://doi.org/10.31788/RJC.2022.1547008
23. Bhupatirajua RV, Battulaa SR, Kapavarapu MVNR, Mandapati VR. Assessment of gas chromatography methodology approach for the trace evaluation of carcinogenic impurity, methyl chloride, in trimetazidine dihydrochloride. Annales Pharmaceutiques Francaises. 2023; 81:64-73. https://doi.org/10.1016/j.pharma.2022.06.012
24. ICH Validation of Analytical Procedures: Text and Methodology Q2(R1); 1994. p. 1-13.
25. ICH guideline, Impurities in New Drug Substances, Q3A (R2), IFPMA, Geneva, Switzerland; 2006.