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Development and validation of a new RP-HPLC method for the determination of process related impurities in pioglitazone hydrochloride

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ABSTRACT

A reversed phase high performance liquid chromatographic method for the determination of process related impurities in pioglitazone hydrochloride was developed and validated. High-quality separation was achieved on a Luna C18 column (150 mm × 4.6 mm, 3 µm) using gradient elution at a flow rate of 1 mL/min and a column temperature of 45 °C. UV detection was performed at 254 nm. The validation was performed according to the International Conference on Harmonization (ICH) guidelines. In addition, the solution stability and method robustness were examined. The method gives satisfactory separation of impurities of pioglitazone hydrochloride and so it is suitable for quantification of the process related impurities as well as for the assay of the active compound.

1. Introduction

Pioglitazone hydrochloride is an oral anti diabetic agent that acts primarily by decreasing insulin resistance. Pioglitazone hydrochloride is used in the management of type 2 diabetes mellitus (also known as non-insulin-dependent diabetes mellitus [NIDDM] or adult-onset diabetes). Pioglitazone hydrochloride selectively stimulates nuclear receptor peroxisone proliferator-activated receptor gamma (PPAR-gamma). Activation of PPAR gamma receptors regulates the transcription of insulin-responsive genes involved in the control of glucose production, transport and utilization. Pioglitazone Hydrochloride, a member of the drug group known as the thiazolidinediones or "insulin sensitizers", is not chemically or functionally related to the alpha-glucosidase inhibitors, the biguanides, or the sulfonylureas. Pioglitazone hydrochloride stimulates the uptake of glucose and fatty acids into cells by promoting the synthesis and expression of cellular glucose and fatty acid transporters [1]. Many studies of pioglitazone demonstrated the improvement of glycemic control, HbA1c, fasting plasma glucose levels, and serum lipid profiles [2,3]. Pioglitazone hydrochloride undergoes extensive hepatic metabolism, predominantly via cytochrome P450 (CYP) 2C8 system. Its elimination half-life was 3-7 h. Pioglitazone and its metabolites were excreted via urine (15-30%). The remainders were excreted into bile and feces [4-6]. More recently, pioglitazone and other active thiazolidinedione have been shown to bind to the outer mitochondrial membrane protein mitoNEET with affinity comparable to that of pioglitazone for PPARγ [7-10]. Pioglitazone hydrochloride reduces insulin resistance in the liver and peripheral tissues; increases the expense of insulin-dependent glucose; decreases withdrawal of glucose from the liver; reduces quantity of glucose, insulin and glycated haemoglobin in the bloodstream.

Literature survey revealed that only few analytical methods have been reported for determining pioglitazone hydrochloride in dosage forms as well as biological fluids. Zhong and Williams reported an analytical method for the simultaneous quantitation of pioglitazone and its metabolites in human serum using HPLC/UV [11]. Yamashita *et al.* have developed a HPLC method for determination of pioglitazone and its metabolites in human serum and urine. In this method separation was achieved by inertsil ODS-2 column (150 × 4.6 mm) with 5 µm particle size, mobile phase consists of phosphate buffer, methanol and acetonitrile; gradient elution was performed at a wavelength 269 nm [12]. Radhakrishna *et al.* reported determination of pioglitazone hydrochloride in bulk and pharmaceutical formulations by HPLC and MEKC

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methods using C18 column (250 × 4.6 mm) 5 µm particle size with a mobile phase consists of phosphate buffer and acetonitrile, detection was carried out by 225 nm [13]. Xue *et al.* have studied a quantitative determination of pioglitazone in human serum by direct-injection HPLC/MS and its application to bioequivalence study [14]. Sane *et al.* have developed a HPLC method for simultaneous determination of pioglitazone and glimipride [15]. Sripalakit *et al.* have reported a HPLC method for the determination of pioglitazone in human plasma using UV detection and its application to a pharmacokinetic study. In this method chromatographic separation was achieved with a RP Apollo C18 Column and mobile phase consisting of methanol, acetonitrile, phosphate buffer, at a wavelength 269 nm [16]. Lofty Saber reported a HPLC method for determination of pioglitazone hydrochloride in tablets [17]. Tahmasebi *et al.* have studied an extraction of trace amounts of pioglitazone with hollow fiber liquid phase micro extraction by HPLC-UV detection in biological fluids [18,19]. Although, to date there is no method reported for determination of process-related substances of pioglitazone hydrochloride in the literature (Figure 1). The present study is aimed at separation and determination of pioglitazone hydrochloride and its process-related impurities by RP-HPLC method [19-21].

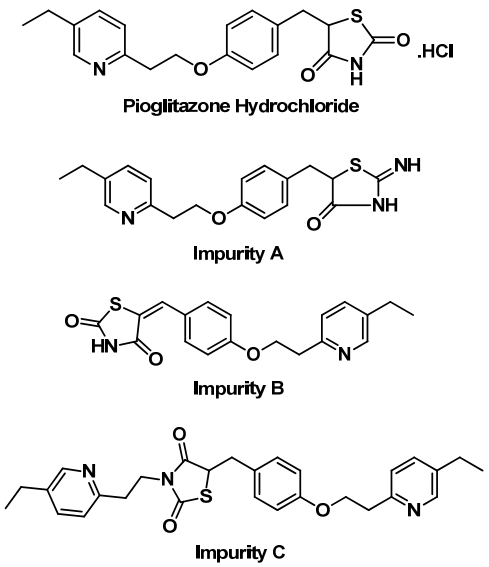

Figure 1. Chemical structures of the pioglitazone hydrochloride and impurities A-C.

2. Experimental

2.1. Instrumentation

An integrated HPLC system with computer based chromatography software (empower) was used. The Water's alliance system with 2695 quaternary low pressure gradient system, auto sampler, column thermostat and photodiode array detector was used for this experiment.

2.2. Chemicals and reagents

Pioglitazone hydrochloride working standard and its process related impurities were synthesized at Pharmazell Research Centre, Visakhapatnam (India) and obtained as gift samples. HPLC grade acetonitrile was obtained from Merck. Analytical grade ammonium acetate, acetic acid was used. High purity water was prepared by using Milli-Q Elix and then using Milli-Q academic purification system (Millipore). 5-(4-(2-(5-Ethylpyridin-2-yl)ethoxy)benzyl)-2-iminothiazolidin-4-one was purchased from (Sigma).

2.3. Preparation of solutions

2.3.1. Preparation of buffer

1.54 g of ammonium acetate was dissolved in 1000 mL of water, pH was adjusted to 4.6±0.05 with acetic acid, filtered through 0.45 µ size filter paper and degassed in an ultrasonic bath.

2.3.2. Preparation of mobile phase

Solvent A: A mixture of acetonitrile and buffer in the ratio (43:57, *v*:*v*) was prepared. Solvent B: A mixture of acetonitrile and buffer in the ratio (80:20, *v*:*v*) was prepared.

2.3.3. Preparation of diluent

A mixture of tetrahydrofuran and methanol in the ratio (1:3, *v*:*v*) was prepared.

2.3.4. Preparation of standard solution

Standard solution was prepared by weighing accurately 50 mg of pioglitazone hydrochloride and transferred into 50 mL volumetric flask containing 30 mL of diluent. The flask was then sonicated for 10 min to dissolve the drug completely and the volume made up to 50 mL with diluent (1 mg/mL).

2.3.5. Preparation of test solution

About 50 mg of sample was weighed accurately and transferred into 50 mL volumetric flask, add 30 mL of diluent, sonicated for 10 min to dissolve the drug completely and the volume was made up to 50 mL with diluent.

2.3.6. Preparation of impurity mixture

About 38 mg of pioglitazone hydrochloride and its process related impurities (impurity A, B and C) were weighed accurately and transferred into 100 mL volumetric flask, 60 mL of diluent was added, sonicated for 10 min to dissolve the compounds and the volume was made up to 100 mL with diluent. 5 mL of this solution was made up to 50 mL with diluent to get a concentration of 38 µg/mL. This impurity stock solution was adequately diluted to study accuracy, precision, linearity, robustness, limit of detection and quantitation.

2.3.7. Chromatographic conditions

To develop a suitable and robust RP-HPLC method for the determination of pioglitazone hydrochloride and its process related impurities, different mobile phases and columns were employed to achieve the best separation and resolution. The method development was initiated with C18 column using a mobile phase containing water and acetonitrile as organic modifier. Broad peaks were observed with this mobile phase. In the above mobile phase water was replaced with phosphate buffer. Peak symmetry was not good. To improve the peak shapes, ammonium acetate buffer was used in place of phosphate buffer. It gave sharp peaks but the problem is impurities are merging. Compounds are having varying polarities. A gradient elution mode was tried; many experiments were conducted by using different gradient programs and columns while optimizing the pH of buffer, buffer concentration, organic modifier strength and wavelength. Finally the best results were observed using a column C18 ODS (150 × 4.6 mm), mobile phase consisting of Solvent A: ammonium acetate buffer and acetonitrile in the ratio (57:43, *v*:*v*).

Table 1. System suitability data.

Name	RT	RRT	Resolution (R_s)	Theoretical plates (N)	Peak symmetry (A_s)
Impurity-A	3.478	0.41	-	2944.3	1.1
Impurity-B	10.770	1.28	5.8	13461	1.0
Impurity-C	18.000	2.15	25.1	122171.6	1.1
Pioglitazone hydrochloride	8.436	1.06	14.1	5622.5	1.0

Table 2. Linearity for impurity-A.

Sample no	Concentration (µg/mL)	Peak area	Mean peak area
1	0.187	6144	6307.6
		6246	
		6533	
2	0.374	12331	12288
		12381	
		12152	
3	0.748	22932	23214.7
		23435	
		23277	
4	1.122	37399	37415.7
		37439	
		37409	
5	1.496	49598	49856.3
		49859	
		50112	
6	1.875	64068	64839.3
		65395	
		65055	
7	2.244	76150	76405.67
		76795	
		76272	
Correlation coefficient		0.9986	
Slope		34558	
Intercept		-1078.9	

Solvent B: ammonium acetate buffer and acetonitrile in the ratio (20:80, *v:v*) at a flow rate of 1.0 mL/min. According to gradient elution program 0-7 min (100% A), 7-20 min (100% B), 20-21 min (100% A). In the above chromatographic conditions gave sharp peaks with minimum tailing, good resolution and shorter runtime for pioglitazone hydrochloride and its process related impurities (Figure 2).

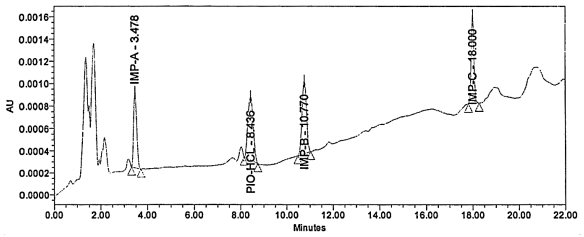

Figure 2. Typical chromatogram for LOQ.

3. Results and discussion

Analytical method validation is a process that demonstrates the suitability of the proposed procedures for the intended purpose. More specifically, it is a matter of establishing documented evidence providing a high degree of assurance with respect to the consistency of the method and results to evaluate the product against defined specifications. The validation parameters viz., specificity, accuracy, precision, linearity, limit of detection, limit of quantitation, robustness, system suitability have to be evaluated as per the ICH guidelines for all analytical methods developed by HPLC.

3.1. System suitability

This is an integral part of development of a chromatographic method to verify that the resolution and reproducibility of the system are adequate enough for the analysis to be performed. It is based on the concept that the equipment, electronics, analytical operations and samples constituting an integral system could be evaluated as a whole. Parameters such as plate number (N), asymmetry or tailing factors (A_s), relative retention time (RRT), resolution (R_s) and reproducibility (% R.S.D), retention time were determined (Table 1). These parameters were determined during the analysis of a "sample" containing the main components and related substances. System suitability parameters were determined and compared with the recommended limits ($1 \geq A_s \leq 2$ and $R_s > 1.5$).

3.2. Specificity

Specificity is the ability of the method to measure the analyte response in presence of its process related impurities. The specificity of the developed HPLC method was performed by injecting blank solution and standard solution spiked with process-related impurities separately. The chromatogram of drug with impurities was compared with the blank chromatogram, to verify the blank interference. No peak was observed at the retention time of pioglitazone hydrochloride and its impurities. Hence the method is specific for the determination of process related impurities in pioglitazone hydrochloride.

3.3. Linearity

Standard solutions at different concentration levels ranging from LOQ to 2.25 µg/mL (150% of specification limit) were prepared and analyzed in triplicate. In order to demonstrate the linearity of detector response for pioglitazone hydrochloride and its impurities, the linearity plot was drawn taking the concentration on X axis and the mean peak area on Y axis. The data was subjected to statistical analysis using a linear-regression model the regression equations and correlation coefficients (r^2) are given in Tables 2-5.

3.3.1. Acceptance criteria

The correlation coefficient should not be less than 0.99.

Table 3. Linearity for impurity-B.

Sample no	Concentration (µg/mL)	Peak area	Mean peak area
1	0.376	8213	8231
		8279	
		8201	
2	0.751	15503	15693.3
		15834	
		15743	
3	1.127	24698	24668.7
		24657	
		24651	
4	1.502	32541	32637.3
		32596	
		32775	
5	1.878	42168	42541.0
		42836	
		42619	
6	2.253	50172	50146.3
		50211	
		50056	
Correlation coefficient		0.9988	
Slope		22684	
Intercept		-831.97	

Table 4. Linearity for impurity C.

Sample no	Concentration (µg/mL)	Peak area	Mean peak area
1	0.377	5964	5851.67
		5980	
		5611	
2	0.754	10844	10990
		10983	
		11143	
3	1.131	17778	17756.7
		17680	
		17812	
4	1.508	23417	23481.7
		23398	
		23630	
5	1.885	29919	30158.7
		30352	
		30205	
6	2.262	35392	35474
		35575	
		35455	
Correlation coefficient		0.9988	
Slope		16017	
Intercept		-515.48	

Table 5. Linearity for pioglitazone hydrochloride.

Sample no	Concentration (µg/mL)	Peak area	Mean peak area
1	0.376	4351	3940.3
		3505	
		3965	
2	0.751	7298	7956
		7904	
		8666	
3	1.127	13099	12814.3
		12461	
		12883	
4	1.502	17290	16787.3
		16485	
		16587	
5	1.878	21674	22979
		24821	
		22442	
6	2.253	27411	27721
		28640	
		27112	
Correlation coefficient		0.9959	
Slope		12780	
Intercept		-1433.5	

3.4. Accuracy and recovery

Accuracy of the test method was determined by analysing pioglitazone hydrochloride drug substance spiked with impurities at three different concentration levels of 50%, 100%, and 150% of each in triplicate at the specified limit. The mean recoveries of all the impurities were calculated (Table 6). The % recovery obtained is well within the limit of 85-115%. This indicated that the method is accurate to determine the process impurities in pioglitazone hydrochloride.

3.4.1. Acceptance criteria

The mean percent recovery of the impurities at each level should be not less than 85.0% and not more than 115.0%.

Table 6. Recovery studies for impurities of pioglitazone hydrochloride.

Name	Spike Level (%)	Concentration spiked (µg/mL)	Concentration recovered (µg/mL)	% Recovery [a]
Impurity-A	50	0.76	0.83	109.2
	100	1.52	1.64	107.9
	150	2.28	2.49	109.2
Impurity-B	50	0.75	0.72	96.0
	100	1.50	1.42	94.7
	150	2.25	2.13	94.7
Impurity-C	50	0.78	0.88	112.8
	100	1.56	1.68	107.7
	150	2.34	2.50	106.8

[a] Average of three determinations.

Table 7. Precision studies for pioglitazone hydrochloride and its impurities.

Name	Method precision %R.S.D (n=6)	Intermediate precision	
		Intraday %RSD (n=6)	Interday %RSD (n=6)
Impurity-A	0.58	0.53±0.09	0.51±0.06
Impurity-B	0.61	0.63±0.05	0.65±0.09
Impurity-C	0.88	0.86±0.07	0.91±0.09
Pioglitazone hydrochloride	0.36	0.39±0.09	0.42±0.07

Table 8. Results for limit of quantitation.

S. No	Name	Concentration (µg/mL)	Observed signal to noise ratio
1	Impurity-A	0.187	9.9
2	Impurity-B	0.376	10.2
3	Impurity-C	0.377	10.4
4	Pioglitazone hydrochloride	0.751	9.8

Table 9. Results for limit of detection.

S. No	Name	Concentration (µg/mL)	Observed signal to noise ratio
1	Impurity-A	0.061	2.9
2	Impurity-B	0.124	3.1
3	Impurity-C	0.124	3.4
4	Pioglitazone hydrochloride	0.250	2.8

3.5. Precision

System precision of the method was evaluated by injecting the standard solution six times and percent relative standard deviation (% R.S.D) for area of pioglitazone hydrochloride peak was calculated. It was found to be less than 2.0% (R.S.D). The precision of the method for the determination of impurities related to pioglitazone hydrochloride was studied for repeatability and intermediate precision at 100% level. Repeatability was demonstrated by analysing the standard solution spiked with impurities for six times. The %R.S.D for peak area of each impurity was calculated. Intermediate precision was demonstrated by analysing same sample of pioglitazone hydrochloride by two different analysts on two different days (Inter-day). Intra-day variations of impurities related to pioglitazone hydrochloride are expressed in terms of % R.S.D Values. Repeatability and intermediate precision for the process-related impurities in Pioglitazone hydrochloride were found to be less than 1.0% R.S.D. The results are given in Table 7, which confirmed good precision of the method.

3.6. Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection (LOD) and limit of quantification (LOQ) represent the concentration of the analyte that would yield signal-to-noise ratios of 3 for LOD and 10 for LOQ, respectively. LOD and LOQ were determined by measuring the magnitude of analytical background by blank samples and calculating the signal-to-noise ratio for each compound by injecting a series of solutions until *S/N* ratio 3 for LOD and 10 for LOQ. LOQ and LOD values are given in Table 8 and 9 and its chromatograms were shown in Figures 2 and 3.

3.7. Robustness

To determine the robustness of the developed method, chromatographic conditions were deliberately altered. The parameters selected were change in flow rate (±0.2 mL/min), change in pH of the buffer (±0.2), change in the ratio of mobile phase (± 4%) and change in the column temperature (±5 °C), the rest of the chromatographic conditions for each alteration study was kept constant. In all the deliberately varied chromatographic conditions, no significant change was observed, which confirmed the robustness of the developed method.

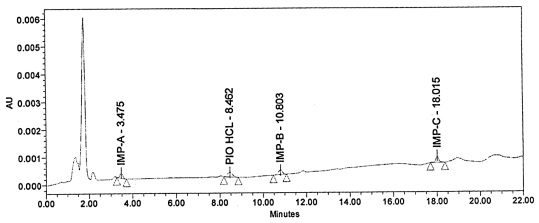

Figure 3. Typical chromatogram for LOD.

4. Conclusion

A new gradient RP-HPLC method was developed for the separation and determination of process related impurities in pioglitazone hydrochloride and validated as per ICH guidelines. The method was found to be simple, selective, precise, sensitive, robust and accurate. Therefore, this method can be used for routine testing as well as stability analysis of pioglitazone hydrochloride drug substance. All statistical results (Mean, %RSD and %recovery) were within the acceptance criteria.

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