

SIMULTANEOUS DETERMINATION OF CLOPIDOGREL AND PIOGLITAZONE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN BULK DRUG AND DOSAGE FORMS

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Abstract. A high performance liquid chromatographic method with UV detection was developed and validated for simultaneous determination of Pioglitazone and Clopidogrel. Separation was performed on a C18 column by isocratic elution with a mobile phase of Methanol: Acetonitrile : Water (80:10:10) at pH 4.6. The UV detection was set at 230 nm. The method proved to be specific, accurate, precise and linear over the concentration ranges of 20-120ppm for both Pioglitazone and Clopidogrel with correlation coefficients always >0.999 for both drugs. The intra-day and inter-day precision and accuracy were less than 2 for both analytes.

Key words: Clopidogrel, Pioglitazone, HPLC-UV method, bioavailability, 230 nm

INTRODUCTION

Clopidogrel is an oral, thienopyridine class antiplatelet agent used to inhibit blood clots in coronary artery disease, peripheral vascular disease, and cerebrovascular disease. It is marketed by Bristol-Myers Squibb and Sanofi under the trade name Plavix. The drug works by irreversibly inhibiting a receptor called P2Y₁₂, an adenosine diphosphate (ADP) chemoreceptor on platelet cell membranes⁽¹⁾. Adverse effects include hemorrhage, severe neutropenia, and thrombotic thrombocytopenic purpura (TTP). Clopidogrel is a prodrug, the action of which may be related to an ADP receptor on platelet cell membranes.

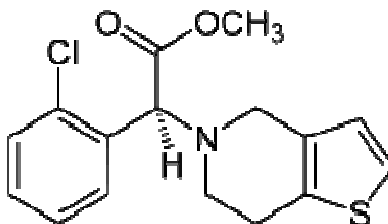


Figure 1: Structure of Clopidogrel

Clopidogrel is indicated for^[2] Prevention of vascular ischemic events in patients with symptomatic atherosclerosis, Acute coronary syndrome without ST-segment elevation (NSTEMI), ST elevation MI (STEMI). Platelet inhibition can be demonstrated two hours after a single dose of oral clopidogrel, but the onset of action is slow, so that a loading-dose of 300 mg is usually administered.

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Serious adverse drug reactions associated with clopidogrel therapy includes, Severe neutropenia, Thrombotic thrombocytopenic purpura (TTP)^[3] Hemorrhage - The annual incidence of hemorrhage may be increased by the co-administration of aspirin.^[4] Gastrointestinal Hemorrhage, Cerebral Hemorrhage (Incidence: 0.1 to 0.4% annually). Use of non-steroidal anti-inflammatory drugs is discouraged in those taking clopidogrel due to increased risk of digestive tract hemorrhage.

Pioglitazone is a prescription drug of the class thiazolidinedione (TZD) with hypoglycemic (antihyperglycemic, antidiabetic) action to treat diabetes. Pioglitazone is marketed as trademarks Actos in the USA, Canada, the UK and Germany, Glustin in Europe, "Glizone" and "Pioz" in India by Zydus Cadila and USV Limited respectively and Zactos in Mexico by Takeda Pharmaceuticals^[5]. Its cardiovascular safety profile compares favorably with rosiglitazone (Avandia), which was withdrawn after concerns about an increased risk of cardiac events. However, pioglitazone has subsequently been found to be associated with bladder tumors and has been withdrawn in some countries.

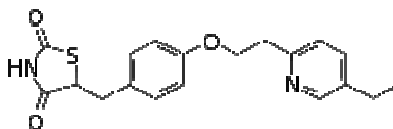


Figure 2: Structure of Pioglitazone

Pioglitazone is used for the treatment of diabetes mellitus type 2 (previously known as non-insulin-dependent diabetes mellitus, NIDDM) in monotherapy and in combination with asulfonylurea, metformin, or insulin. Pioglitazone has also been used to treat non-alcoholic steatohepatitis (fatty liver), but this use is presently considered experimental.^[6] Pioglitazone has also been found to reduce the risk of conversion from prediabetes to diabetes mellitus type 2 by 72%.^[7]

Pioglitazone can cause fluid retention and peripheral edema. As a result, it may precipitate congestive heart failure (which worsens with fluid overload in those at risk). It may cause anemia. Mild weight gain is common due to increase in subcutaneous adipose tissue. In studies, patients on pioglitazone had an increased proportion of upper respiratory tract infection, sinusitis, headache, myalgia and tooth problems. The risk of hypoglycemia is low in the absence of other drugs that lower blood glucose.

MATERIALS AND METHODS

Chemicals and reagents

Reagents used like Acetonitrile, Methanol, Water which are of HPLC grade were purchased from E.Merck, Mumbai, India

Instrumentation and analytical conditions

The analysis of the drug was carried out on Shimadzu HPLC model (VP series) containing LC-10AT (VP series) pump, variable wave length programmable UV/visible detector SPD-10AVP and rheodyne injector (7725i) with 20µl fixed loop. Chromatographic analysis was performed

using Inertsil ODS C-18 column with 250 x 4.6mm internal diameter and 5 μ m particle size. Shimadzu electronic balance (AX-200) was used for weighing. Isocratic elution with Acetonitrile: Methanol 10:90 (V/V) was selected with a flow rate of 1.0 ml min⁻¹. The detection wavelength was set at 235 nm with a runtime of 10 min. The mobile phase was prepared freshly and it was degassed by sonicating for 5 min before use. The column was equilibrated for at least 30min with the mobile phase flowing through the system. The column and the HPLC system were kept at ambient temperature.

Preparation of Stock, working standard solutions and Sample solutions

Standard solutions of concentration 1000 μ g/mL of Pioglitazone and 1000 μ g/mL of Clopidogrel were prepared separately using methanol. From standard solutions further solution are prepared by using methanol.

The formulation tablets of Pioglitazone (ACTOS -45mg) and Clopidogrel (Plavix -75mg) were crushed to give finely powdered material. Powder equivalent to 10 mg of drug was taken separately in 10 ml of volumetric flask containing 5 ml of Methanol and was shaken to dissolve the drug and then filtered through Ultipor N₆₆ Nylon 6,6 membrane sample filter paper. Volume of the filtrate was adjusted to the mark with the same solvent to obtain concentration of 80 ppm. 1ml from the each of the two drug solutions were taken and mixed well and injected into HPLC system and the resultant assay was calculated based on the peak areas.

Optimization of HPLC Method

All drugs were subjected to chromatographic analysis using mobile phases of differing pH, flow rate using the under mentioned chromatographic conditions. The changes in the retention time of all drugs were noted as a function of changing mobile phase, pH, flow rate, strength and selectivity. Initially methanol: water in the ratio of (80: 20) was tried but both the peaks merged. Later methanol: water: Acetonitrile in the ratio of (80: 10:10) was tried and it was found that both the peaks were well separated with acceptable resolution. Hence methanol: water: Acetonitrile in the ratio of (80: 10:10) at flow rate of 1.2 mL/min was finalized which gave acceptable retention time, plates and good resolution for Pioglitazone and Clopidogrel (Figure:3)

Validation of the method

Validation of the optimized HPLC method was carried out with respect to the following parameters.

Linearity and range: The mixed standard solution (1000 μ g/mL of Pioglitazone and 1000 μ g/mL of Clopidogrel) was further diluted to get Pioglitazone and Clopidogrel concentration in the range of 20-120 μ g/mL. Linearity of the method was studied by injecting six concentrations of the drug prepared in the mobile phase keeping the injection volume constant. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs.

Precision: The precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analysis of concentration 80ppm for Pioglitazone and 80ppmL for Clopidogrel six times on the same day. The intermediate precision of the method was checked by repeating studies on three different days.

Limit of detection and limit of quantitation: Limits of detection (LOD) and quantification (LOQ) represent the concentration of the analyte that would yield signal-to-noise ratios of 3 for

LOD and 10 for LOQ, respectively. To determine the LOD and LOQ, serial dilutions of mixed standard solution of Pioglitazone and Clopidogrel was made from the standard stock solution. The samples were injected in LC system and measured signal from the samples was compared with those of blank samples.

Robustness of the method: To evaluate robustness of a HPLC method, few parameters were deliberately varied. The parameters included variation of flow rate, percentage of mobile phase and wavelength. Robustness of the method was done at three different concentration level 80 µg/mL for Pioglitazone and Clopidogrel.

Specificity: The specificity of the method towards the drug was established through study of resolution factor of the drug peak from the nearest resolving peak. The peak purity of Pioglitazone and Clopidogrel was determined by comparing the spectrum at three different regions of the spot i.e. peak start (S), peak apex (M) and peak end (E). Effect of excipients of formulation was studied for whether it interfered with the assay.

Accuracy: Accuracy of the method was carried out by applying the method to drug sample to which know amount of Pioglitazone and Clopidogrel standard powder corresponding to 50, 100 and 150 % of label claim had been added (Standard addition method), mixed and the powder was extracted and analyzed by running chromatogram in optimized mobile phase.

H.P.L.C CONDITIONS		Result
ELUTION		Isocratic
A.P.I	CONC	80µg/ml
MOBILE PHASE		MEOH 80%, ACN 10%, Water 10 %
PH		4.6
COLUMN		C18, 250 ×4.6 mm
WAVE LENGTH		230nm
FLOW		1.2 ml/min
RUNTIME		12 Min
RETENSION TIME	Pioglitazone	5.31 min
	Clopidogrel	9.26min
AREA	Pioglitazone	461445
	Clopidogrel	655822
TH.PLATES	Pioglitazone	2144
	Clopidogrel	9166
TAILING FACTOR	Pioglitazone	0.92
	Clopidogrel	0.94
PUMP PRESURE		10.5mpa

Analysis of a marketed formulation: To determine the content of Pioglitazone and Clopidogrel conventional tablets (Brand name: ACTOS, Label claim: 45 mg Pioglitazone and Brand name: Plavix, Label claim: 75 mg Clopidogrel), twenty tablets were weighed; their mean weight determined and finely powdered separately. The weight of the tablet triturate equivalent to 10 mg of Pioglitazone and 10 mg Clopidogrel was transferred into a 10 mL volumetric flask containing 5 mL methanol, sonicated for 30 min and diluted upto 10 mL with methanol. The resulting solution was centrifuged at 3000 rpm for 5 min and the drug content of the supernatant was determined. Supernatant was taken and after suitable dilution the sample solution was then filtered using 0.45-micron filter (Millipore, Milford, MA). The above stock solution was further diluted to get sample solutions 80 µg/mL for Pioglitazone and Clopidogrel separately. 1ml from the each of the two drug solutions were taken and mixed well and injected into HPLC system. The peak areas were measured at 230 nm and concentrations in the samples were determined.

RESULTS AND DISCUSSION

The results of validation studies on simultaneous estimation method developed for Pioglitazone and Clopidogrel in the current study involving methanol: water: ACN (80: 10:10) are given below.

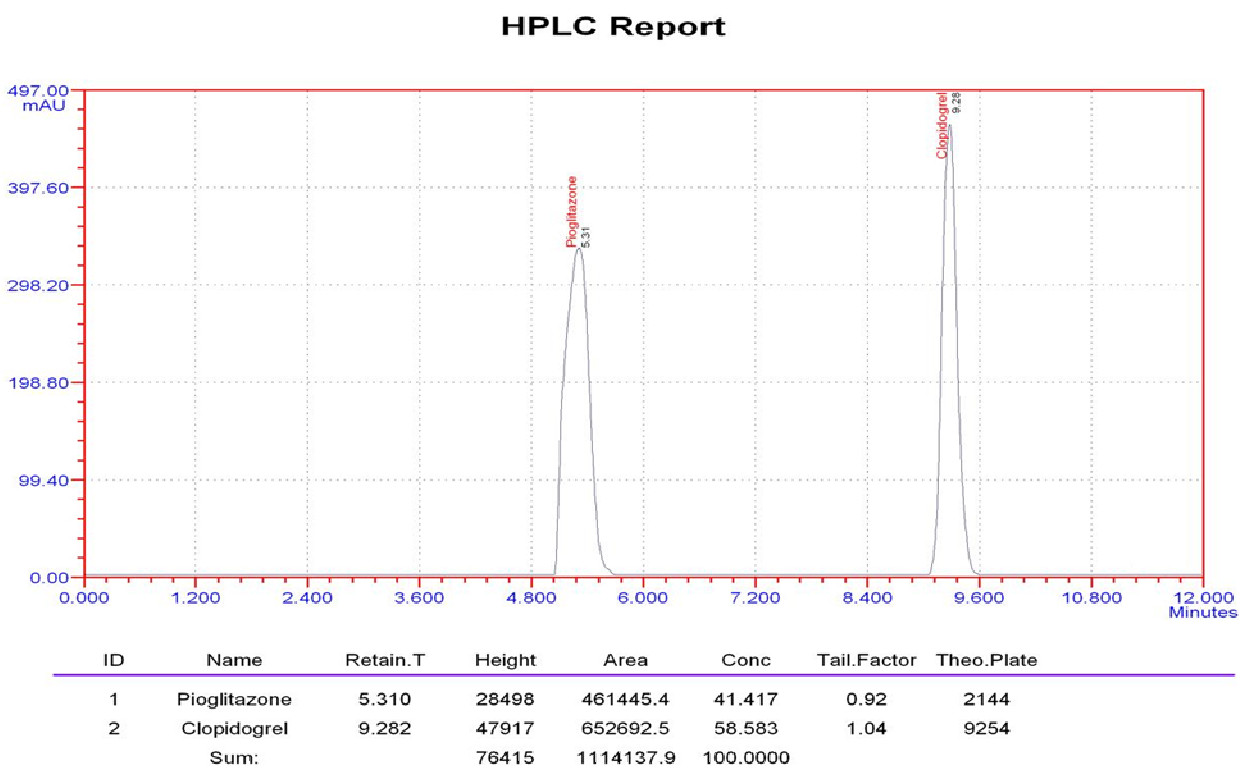


Figure 3: Standard Chromatogram of Pioglitazone and Clopidogrel

Linearity: Pioglitazone and Clopidogrel showed good correlation coefficient ($r^2 = 0.999$ for Pioglitazone and 0.999 for Clopidogrel) in given concentration range (20-120 $\mu\text{g/mL}$ for both Pioglitazone and Clopidogrel). The values of the slope and intercept were 0.000174 and -1.004 for Pioglitazone and 0.000125 and -1.454 for Clopidogrel respectively.

S.No	Concentration	Area	
		Pioglitazone	Clopidogrel
1	20	121936	182890
2	40	238933	331453
3	60	363508	496555
4	80	461445	655822
5	100	570571	828756
6	120	699126	958549
Result	Correlation Coefficient	0.999	0.999
	Intercept	-1.004	-1.454
	Slope	0.000174	0.000125

Table 1: Linearity results

Precision: The results of the repeatability and intermediate precision experiments are shown in Table 1. The developed method was found to be precise as the RSD values for repeatability and intermediate precision studies were $< 2\%$, respectively as recommended by ICH guidelines 13-15.

S.No	Concentration	Area	
		Pioglitazone	Clopidogrel
1	80ppm	447759	529153
2		437939	533459
3		438960	534915
4		431317	533698
5		441893	529392
6		447676	532860
Result	RSD	1.49	0.45

Table2: Intraday Precision results

S.No	Concentration	Area	
		Pioglitazone	Clopidogrel
1	80ppm	446668	518370
2		441289	522659
3		438845	515640
4		440495	515832
5		431317	516518
6		444710	518696
Result	RSD	1.21	0.58

Table3: Interday Precision results

LOD and LOQ: Signal-to-noise ratios of 3:1 and 10:1 were obtained for the LOD and LOQ respectively. The LOD and LOQ were found to be 0.25µg/mL and 0.825 µg/mL for Pioglitazone and 0.1 µg/mL and 0.33 µg/mL for Clopidogrel, respectively.

Robustness of the method: Each factor selected (except columns from different manufacturers) was changed at three levels (-0.1, 0 and 0.1). One factor at the time was changed to estimate the effect. Thus, Robustness was performed under small changes of three chromatographic parameters (factors). Insignificant differences in peak areas were observed (Table 4).

Parameter	Change	Area of Pioglitazone	Area of Clopidogrel	% Change of Pioglitazone	% Change of Clopidogrel
Standard	461445	655822
Mobile Phase	MEOH 70%, ACN 15%, Water 15 %	455802	648818	1.22	1.07
	MEOH 90%, ACN 5%, Water 5 %	457771	656699	0.8	0.13
Wavelength	225nm	454400	650399	1.53	0.83
	235nm	462918	658404	0.32	0.39
Flow Rate	1.4ml/min	463989	646741	0.55	1.37
	1.00ml/min	455224	658455	1.35	0.40

Table 4: Robustness results

Recovery: As shown from the data in Table 3 good recoveries of the Pioglitazone and Clopidogrel in the range from 98.34 to 101.28 % were obtained at various added concentrations.

% of Recovery	Target Conc., (ppm)	Spiked conc, (ppm)	Final Conc, (ppm)	Conc of Pioglitazone Obtained	Conc of Clopidogrel Obtained	% of Assay of Pioglitazone	% of Assay of Clopidogrel
	50%	40	20	60	59.62	59.94	99.03
40		20	60	59.65	59.70	99.70	99.5
40		20	60	59.17	59.60	98.62	99.33
100%	40	40	80	79.75	78.70	99.69	98.45
	40	40	80	78.67	79.05	98.34	98.81
	40	40	80	78.99	80.21	98.74	100.27
150%	40	60	100	99.00	98.23	99.00	98.23
	40	60	100	100.99	100.25	100.99	100.25
	40	60	100	101.28	99.95	101.28	99.95

Table 5: Recovery results

Analysis of a formulation: Experimental results of the amount of Pioglitazone and Clopidogrel in tablets, expressed as a percentage of label claims were in good agreement with the label claims thereby suggesting that there is no interference from any of the excipients which are normally present. The drug content was found to be 98.52% for Pioglitazone and 99.52 % for Clopidogrel. Two different lots of Pioglitazone and Clopidogrel tablets were analyzed using the proposed procedures as shown in Table 6.

Drug	Formulation	Dosage	Concentration	Amount found	% Assay
Pioglitazone	ACTOS	45mg	80 ppm	78.82	98.52
Clopidogrel	PLAVIX	75mg	80 ppm	79.62	99.52

Table 6: Formulation results

CONCLUSION

HPLC method was developed and validated as per ICH guidelines. UV detection allowed an accurate quantitation of chromophoric compounds. The drug was analyzed by HPLC method using Chromosil C18, 250 x4.6 mm with isocratic conditions and simple mobile phase

containing MEOH 80%, ACN 10%, Water 10 % at flow rate of 1.2 mL/min using UV detection at 230 nm. The procedure has been evaluated for the linearity, accuracy, precision and robustness in order to ascertain the suitability of the analytical method. The method was also applied to marketed samples. It has been proved that the method is selective and linear between concentration range 20-120 µg/mL for both Pioglitazone and Clopidogrel. The LOD and LOQ were found to be 0.25µg/mL and 0.825 µg/mL for Pioglitazone and 0.1 µg/mL and 0.33 µg/mL for Clopidogrel, respectively. Statistical analysis proves that the method is suitable for the analysis of Pioglitazone and Clopidogrel as bulk drug and in pharmaceutical formulation without any interference from the excipients. It may be extended to study the degradation kinetics of Pioglitazone and Clopidogrel and also for its estimation in plasma and other biological fluids.

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