How to Cite:
Hephzibah, K., & Shanmugasundaram, S. (2022). Validated stability-indicating RP-HPLC method for simultaneous estimation of candesartan and pioglitazone in human plasma. *International Journal of Health Sciences*, *6*(S6), 10226–10239. https://doi.org/10.53730/ijhs.v6nS6.12718

Validated stability-indicating RP-HPLC method for simultaneous estimation of candesartan and pioglitazone in human plasma

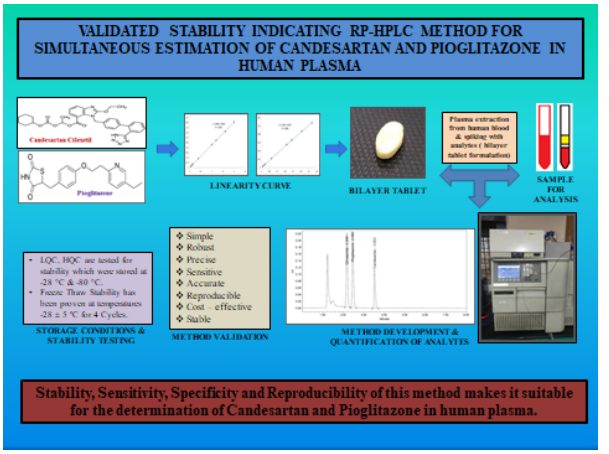
Kola Hephzibah
Research Scholar, Department of Pharmaceutical Analysis, SRM College of Pharmacy, SRMIST, Kattankulathur, Chennai, Tamil Nadu – 603203
Email: kh7475@srmist.edu.in

Dr. Sangeetha Shanmugasundaram
Professor, Department of Pharmaceutics, SRM College of Pharmacy, SRMIST, Kattankulathur, Chennai, Tamil Nadu – 603203
*Corresponding author email: sangeets2@srmist.edu.in

Abstract---Objective: The focus of this study was to establish a simple, robust, and validated RP-column approach to analyze Candesartan and Pioglitazone in a bilayer tablet dosage form. To broaden patient compliance in conditions such as diabetes and hypertension, a combination of drugs is often prescribed. As a result, a bilayer tablet containing a fixed-dose combination of 8 mg of Candesartan and 15 mg of Pioglitazone was developed. Both drugs were analyzed and chromatographically separated using the liquid chromatography technique. Methodology: waters 2695 with a quaternary pump and Inertsil C18 column mobile phase as buffer: acetonitrile (60:40) and flow rate of 1ml per minute and detection at 220nm was used for performing Chromatography. Results: Internal standard, Candesartan, and Pioglitazone eluted with retention times of 2.208, 2.569, and 3.553 minutes, respectively. There was no interference found between the peaks. With a correlation value of 0.999, the approach is verified across a linear range of 0.5 µg/ml – 20.0µg/ml for Candesartan and 0.08 µg/ml – 3.2 µg/ml for Pioglitazone. 6 samples at LLOQ level were analyzed for accuracy and precision and found to be in limit and all the analytes were stable at 28 °C and -80 °C in human plasma. Conclusion: This approach is appropriate for determining Candesartan and Pioglitazone in human plasma due to its reliability, sensitivity, precision, and constancy.

Keywords---candesartan, pioglitazone, glimepiride, human plasma, RP-HPLC.

Graphical abstract



Introduction

Diabetes mellitus (Type 2 diabetes) makes up for 95% of all diabetes cases and is on the rise. The consequences of this condition will substantially shorten people's lives.(1,2) since hypertension is usually associated with type 2 diabetes, treatment of hypertension in addition to glycemic management, is crucial in reducing cardiovascular events in people with T2DM. (3,4)Hence numerous hypertensive patients are treated with combination of antihypertensive and antidiabetic drugs(5). A combination of Candesartan and Pioglitazone is formulated as an oral bilayer tablet for the treatment of type 2 diabetes. Pioglitazone is a thiazolidinedione that is used in tandem with healthy eating and exercise to normalize glycemic levels in individuals with type 2 diabetes. (6) PPAR-gamma is a nuclear receptor activated by Pioglitazone (7)Treatment with any of several available Ang II receptor blockers (ARBs), agents that bind antagonistically to the Ang II, type I receptor (AT1R), resulted in renoprotection, according to clinical and basic research investigations. (8–10)

This one ARB, Candesartan which is routinely taken to decrease blood pressure, has been unambiguously established to enhance renal function and ameliorate renal disease. (11,12).A review of the literature concluded that a few techniques, such as Ultra Performance Liquid Chromatography(13,14), High-Performance Liquid Chromatography(15,16), and Ultraviolet Spectroscopy (17,18), have been published for quantifying candesartan and pioglitazone bio analytically. This pairing was not evaluated in human plasma, according to the review. hence, an initiative has been taken to develop a simple, swift, and reproducible RP-HPLC procedure for quantification in human plasma using Glimepiride as an internal standard for this combination. The new approach was validated in accordance with USFDA requirements. The current bioanalytical approach's purpose is to show that it is reasonable for the justification and so would be beneficial for pharmacokinetic experiments.

Experimental
Chemicals Used

Madras Pharmaceuticals, Chennai, Tamil Nadu, provided candesartan and pioglitazone as gift samples. Sigma-Aldrich, Chennai, India, provided acetonitrile, methanol, and other chemicals which are of HPLC grade. Throughout the investigation, HPLC-grade water from the Milli-Q water purification system was utilized.

Instrumentation

Waters 2695 HPLC integrated with a quaternary pump, high-speed autosampler, column oven, degasser, and 2996 PDA detector with class Empower-2 software has been used for chromatography.

Condition for Chromatography

The separation was accomplished using an Inertsil C18 column with a mobile phase of % 0.1OPA and acetonitrile (60:40). The separation was observed for 10 minutes at 220 nm with 1ml/min flow rate. The sample is diluted using a water:acetonitrile (50:50) diluent ratio.

Internal standard

50 mg of Glimepiride is diluted to 500µg/ml in diluent. Take 0.5ml of this solution and drop it in a 10 ml volumetric flask. Fill the rest of the flask with diluent to make 25µg/ml solutions. Take 0.5ml of the above solution and spike blank plasma with working stock dilutions of analyte to create a 5µg/ml ISD concentration.

Linearity curve samples and quality control samples

Individual stocks of Pioglitazone and Candesartan are made by dissolving 25 mg of Pioglitazone and 4 mg of Candesartan in diluent to obtain 100µg/ml and 16µg/ml, respectively. To generate calibration curve standards, they were further diluted for spiking in plasma by diluent. The spiked samples for are obtained by taking into 10 ml volumetric flasks and adjusting the volume with diluent. Pioglitazone had a working concentration of 0.5 to 20.0 µg/ml and Candesartan had a range of 0.08 to 3.2 µg/ml. Calibration standards and quality control (QC) samples were made by spiking blank plasma with analyte working stock dilutions to achieve the desired concentrations.

Sample preparation by plasma spiking

Add 750µl of plasma and 500µl of internal standard, 250µl of both spiking solutions to centrifuge tube with 1 ml of Acetonitrile, and operate till 15 seconds on the cyclomixer. Then vertex for 2 minutes before centrifuging for 5 minutes at 3200 rpm. After centrifugation, collect and filter the sample. The resulting organic layer was analysed.

Methodology

Following the USFDA requirements, a rigorous and extensive validation approach had been used to carryout validation for various parameters and stability studies.

Specificity

Six blank standards and LLOQ samples are used to test the specificity and screening of biological matrix. All were examined to see how much interference the plasma had with Candesartan, pioglitazone, and Glimepiride.

Linearity curve

A investigation of standard plots associated with an eight-point standard calibration curve was used to evaluate the method's linearity. LLOQ was present in the concentrations of the eight analytes. The calibration curve is constructed by plotting the analytes' peak area ratio to the internal standard against standard concentrations.

Accuracy, precision

Accuracy & precision is examined in six duplicates for analytes at low, medium, higher, and LLQ and QC samples whereas precision,accuracy are measured for 3 days using QC samples. Over these batches, mean is estimated and quantification of analytes was done and accuracy, precision were computed.

Recovery

The analytes were recovered from the extraction technique at the LQC, MQC, and HQC levels and assessed by comparing the peak areas spiked prior to extraction and quality control working solutions spiked with extracted plasma.

Sensitivity

Sensitivity was determined using an LLOQ level sample in six duplicates.

Stability

Zero-hour, freeze-thaw, and long-term stability tests are done in -28 °C and -80 °C with 6 duplicates of HQC and LQC level samples on day zero. stability of at-28 °C was tested by preserving samples for 37 days.Samples are thawed and tested immediately after being kept at -80 oC. The acquired results are compared to those obtained by reprepared new samples. percent mean accuracy & percent coefficient of variation were computed for free-thaw stability utilising LQC and HQC level samples.

Results and Discussion

Method optimization

Waters 2695-quaternary pump-Inertsil C18 column mobile phase as buffer: acetonitrile (60:40) and 1ml per minute flow rate and detection at 220nm is used for performing Chromatography produced best results. The retention times for Pioglitazone and Candesartan were 2.569 and 3.553, respectively, under these settings.

Validation
System suitability and autosampler carry over

MQC level sample is injected six times homogeneously to test system suitability. For retention time and response, the percent coefficient of variation was estimated. Table 1 summarises the findings. The acquired results are < 1%, demonstrating the system's feasibility for measurement of chosen combos in human plasma. ULOQ (upper limit of quantification) and LLOQ sample were injected into Autosampler to verify that accuracy and precision were not affected and no indication of carryover is observed.

Table 1
Statistical analytical data of parameters measured for system suitability

specifications	Candesartan	Pioglitazone	Glimepiride	Approval criterion
Retention time	0.19	0.21	0.5	Relative Standard Deviation ≤ 2
Area under peak	0.23	0.86	0.37	Relative Standard Deviation ≤ 5
Resolution	8.5	2.7		Rs>2
Number of theoretical plates (N)	34505.67	148105	625333.7	Directly proportional to separation efficiency
Tailing Factor (T)	1.2	1.2	1.3	T ≤ 2
Height Equivalent to a Theoretical Plate (cm/plate)	6738.1	4578.7	3904.8	Smaller the value, higher the column efficacy

Specificity

When chromatograms of blank and quality control samples, no endogenous source of interference was found in all batches as observed in Figures 2 and 3, respectively. This demonstrates the method's sensitivity to candesartan and pioglitazone.

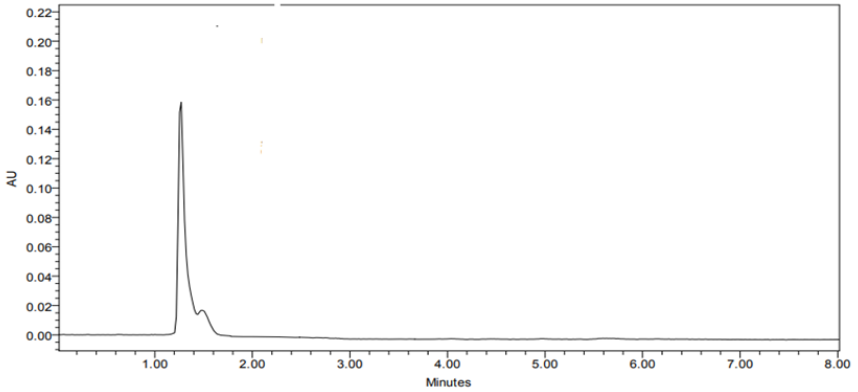


Fig 1. Elution in human plasma(blank)

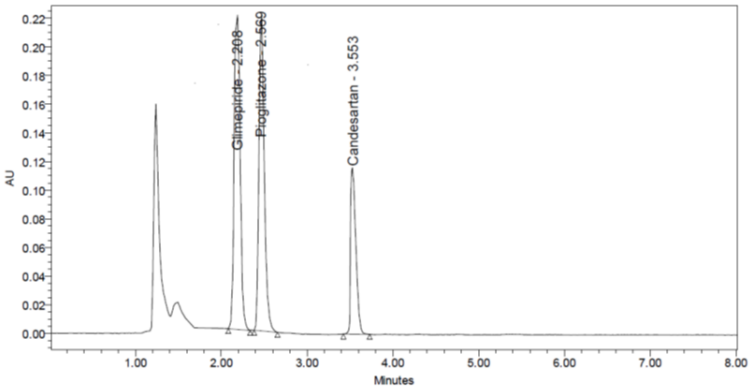


Fig 2. Elution in human plasma spiked with Candesartan, Pioglitazone, and Glimepiride

Linearity

Calibration curve was built using the analyte's peak area to the internal standard ratio. The calibration curve's most variable regression equation for Candesartan and Pioglitazone was y = 0.33814x + 0.00031and y = 0.22684x + 0.00048, respectively. Correlation coefficient of 0.999, verified calibration graph's linearity. Figures 3,4 and 5 show the standard curves for Candesartan and Pioglitazone.

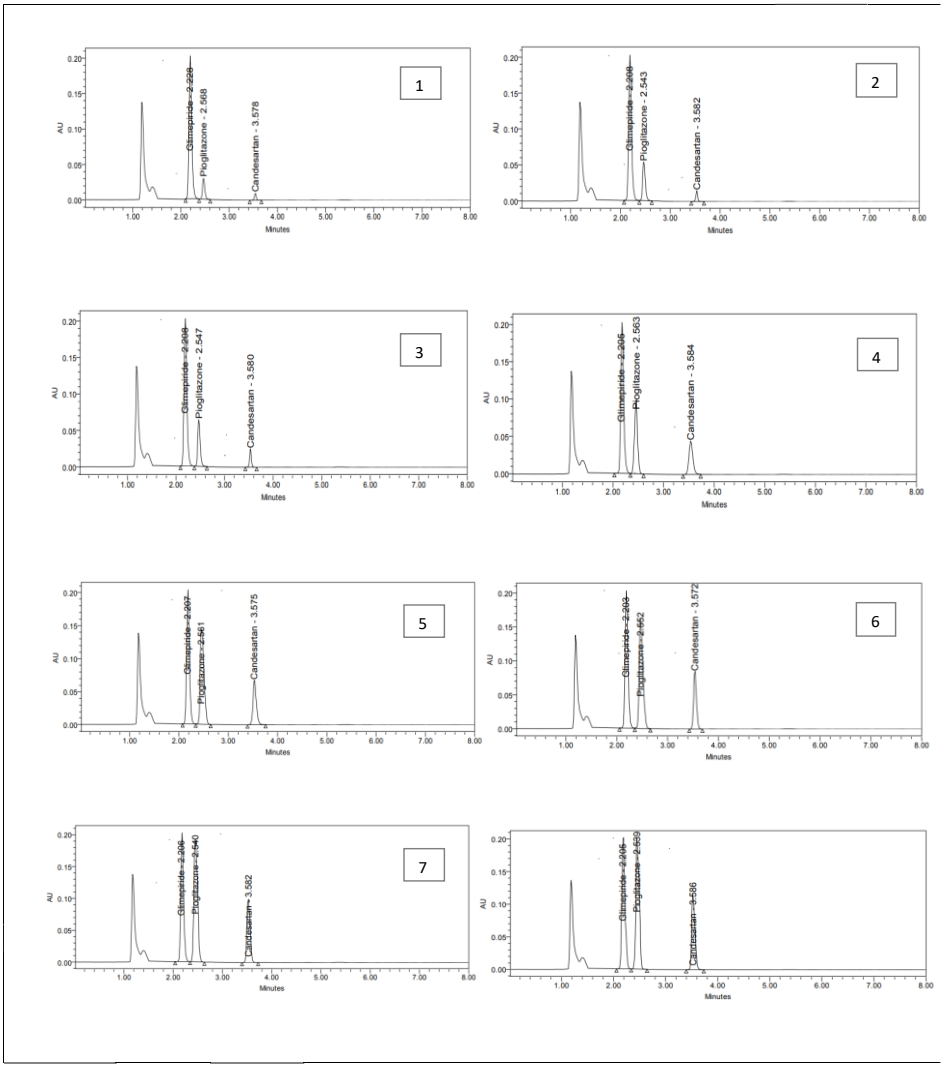


Fig 3. Linearity chromatograms of Pioglitazone and Candesartan

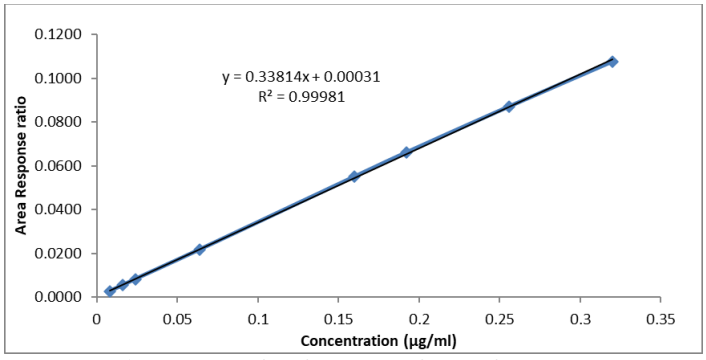


Fig 3. Standard curve of Candesartan

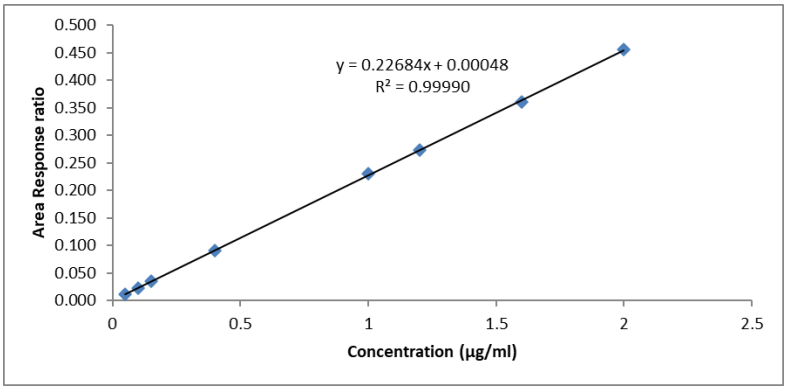


Fig 4. Standard curve of Pioglitazone

Precision and accuracy

Accuracy is computed by calculating the % mean accuracy and precision by calculating standard deviation relatively from fig.4 and summarized in table 2. The obtained findings show an adequate accuracy for all amounts evaluated in both intra-day and inter-day samples.

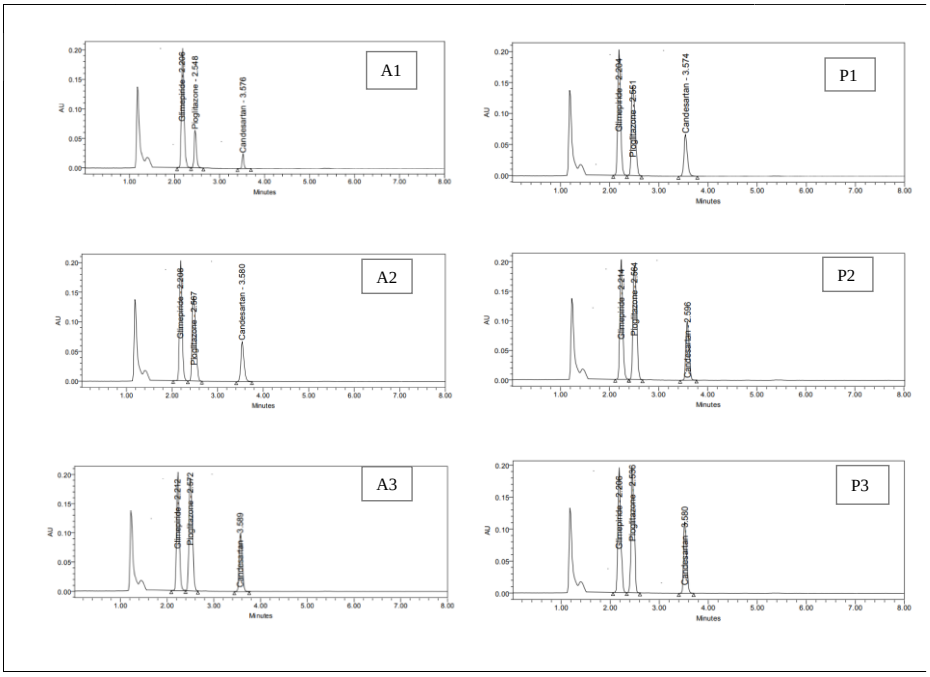


Fig. 4. A1,A2,A3 are accuracy chromatograms and P1,P2,P3 are precision chromatograms

Table 2a precision and accuracy findings of CANDESARTAN			
	mean±SD	%CV	%mean recovery
DAY 1			
Nominal conc. (ng/ml)			
8	8.0253±0.12919	1.61	100.32
24	24.2693±0.55777	2.3	101.12
160	159.7500±1.00349	0.63	99.84
256	256.0000±1.26491	0.49	100
DAY 2			
8	7.9907±0.09572	1.2	99.88
24	24.2733±0.53403	2.2	101.14
160	160.2868±0.76707	0.48	100.18
256	256.0667±0.74744	0.29	100.03
BETWEEN BATCH			
8	8.0309±0.10101	1.26	100.39
24	24.2753±0.50593	2.08	101.15
160	160.2158±0.99477	0.62	100.13
256	256.1000±0.85612	0.33	100.04

Table 2b Precision and accuracy findings of PIOGLITAZONE			
	mean±SD	%CV	%mean recovery
DAY 1			
Nominal conc. (ng/ml)			
50	49.6867±0.51679	1.04	99.37
150	149.56.3±0.1.05289	0.7	99.71
1000	999.0047±4.18706	0.42	99.9
1600	1599.4998±4.83967	0.3	99.97
DAY 2			
50	7.9907±0.09572	1.2	99.88
150	24.2733±0.53403	2.2	101.14
1000	160.2868±0.76707	0.48	100.18
1600	256.0667±0.74744	0.29	100.03
BETWEEN BATCH			
50	49.5755±0.51125	1.03	99.15
150	149.5048±0.85645	0.57	99.67
1000	1000.1714±2.96950	0.3	100.02
1600	1600.2675±3.01202	0.19	100.02

Recovery

The peak area of quality control samples were compared to peak area of unextracted quality control samples at the same level to assess Candesartan and Pioglitazone recovery from fig.5. Table 3 summarises the findings of the recovery research. The findings are within the acceptable range. At each QC level, the allowable limit was percent CV of recovery, and the IS limit should be 15.00 percent. For all QC levels, the total mean recovery percent CV should be 20

percent. The resulted data demonstrated efficient extraction for the improved approach.

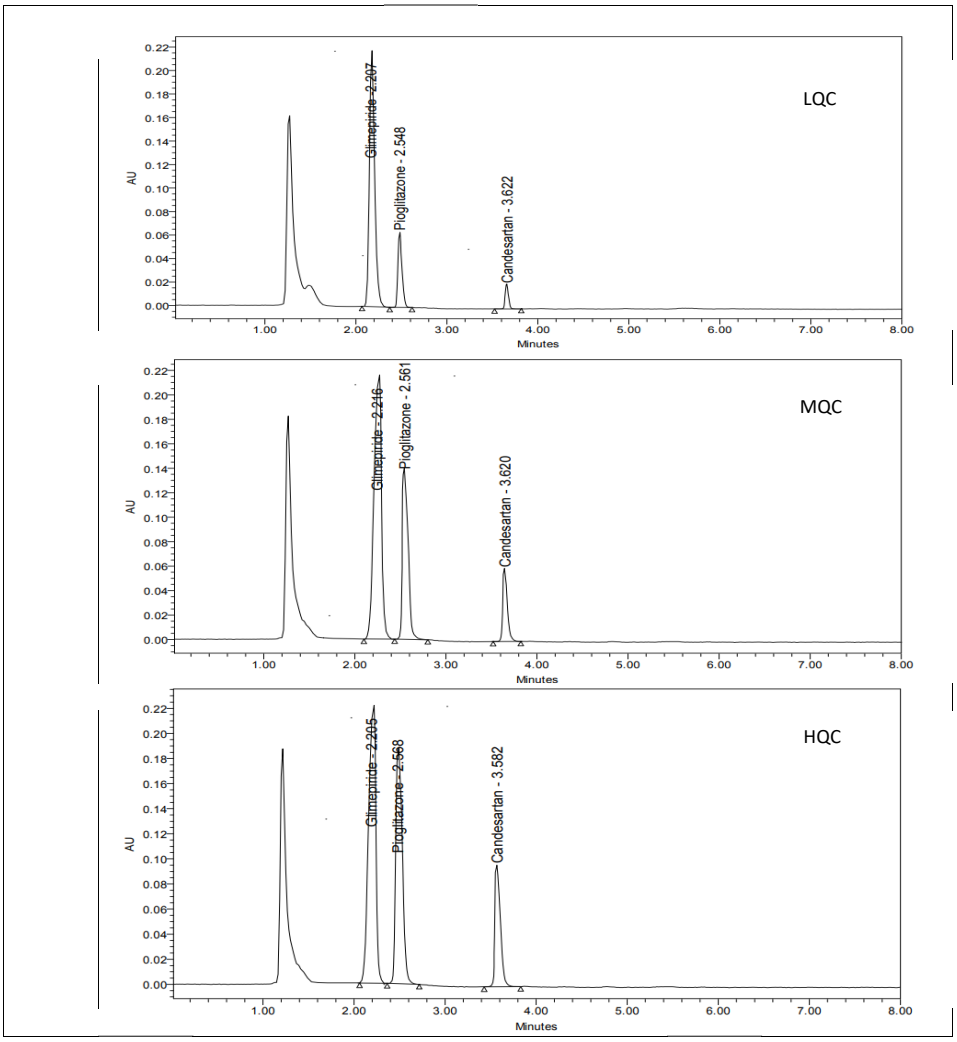


Fig. 5. LQC, MQC, HQC chromatograms

Table 3 Recovery data from human plasma		
	concentration (µg/ml)	% Mean Recovery
CANDESARTAN	24.0 (LQC)	99.14
	160.00(MQC)	99.07
	256.00(HQC)	99.11
	overall mean recovery	99.105
PIOGLITAZONE	150.00(LQC)	98.38
	1000.00(MQC)	99.28
	1600.00 (HQC)	98.72

	overall mean recovery	98.8

Stability

Six duplicates of quality control samples at low-high levels were analyzed at room temperature for 24 hours to assess analyte stability in human plasma (day zero). The amounts observed are compared to those of newly produced and processed samples. The results showed that Candesartan and Pioglitazone are stable in human plasma for at least 24 hours when kept at room temperature. The freeze-thaw stability of plasma samples-Candesartan and Pioglitazone were investigated through 3 cycles of freeze-thawing, which included thawing at ambient temperature for 2 to 3 hours and again frozen for 12 to 24 hours. The findings from Table 4 suggest the stability of analytes in human plasma.

Table 4
Stability of Candesartan and Pioglitazone in human plasma

Stability conditions	CANDESARTAN		PIOGLITAZONE	
	LQC	HQC	LQC	HQC
	24	256	150	1600
Day zero				
Mean calculated concentration (µg/ml)±SD*	24.5182±0.62678	255.982±0.65717	149.5607±0.83929	1600.3092±0.46534
% CV	2.56	0.26	0.56	0.03
% Mean accuracy	102.16	99.99	99.71	100.02
Freeze-thaw stability (4 cycles)				
Mean calculated concentration (µg/ml)±SD*	24.065±0.65841	256.5983±6.19193	149.9567±0.36407	1614.1383±21.03025
% CV	2.74	2.41	0.24	1.3
% Mean accuracy	100.27	100.23	99.97	100.88
Stability (-28 °C)				
Mean calculated concentration (µg/ml)±SD*	24.3193±0.49429	256.2867±0.53884	149.5987±0.67109	1595.5837±3.60889
% CV	2.03	0.21	0.45	0.23
% Mean accuracy	101.33	100.11	99.73	99.72
Stability (-80 °C)				
Mean calculated concentration (µg/ml)±SD*	24.4471±0.73392	256.3200±0.66531	150.9073±0.6454	1600.2932±602.60509

			4	
% CV	3	0.26	0.43	37.66
% Mean accuracy	101.86	100.13	100.6	100.02

Conclusion

The suggested approach for estimating the binary combination of Candesartan and Pioglitazone in human plasma is simple, precise, and consistent. The technique is cost efficient and ideal for analyzing a large number of samples due to the easy precipitation of protein, duration of 10 minutes, and isocratic elution. The approach has been validated in accordance with the requirements of the US-FDA. The approach may be inferred to be suitable for regular measurement of Candesartan and Pioglitazone in human plasma.

Acknowledgment

We are grateful to SRM College of Pharmacy, SRMIST , kattankulathur , Chennai , Tamil Nadu for supporting this work.

Authors contributions

Experimental design, execution, data generation and writing of manuscript were done by KOLA HEPHZIBAH. The design, guidance for work and manuscript review was done by Dr. (Mrs.) SANGEETHA SHANMUGASUNDARAM.

Conflict of interests
Declared none

References

1. Al-Majed A, Bakheit AHH, Abdel Aziz HA, Alharbi H, Al-Jenoobi FI. Pioglitazone. Profiles Drug Subst Excipients Relat Methodol. 2016;41:379–438.
2. Amanda I Adler, Irene M Stratton, H Andrew W Neil, John S Yudkin, David R Matthews, Caroe A Cull, et al. Association of systolic blood pressure with macrovascularand microvascular complications of type 2 diabetes(UKPDS 36): prospective observational study.
3. Brenner BM, Cooper ME, De Zeeuw D, Grunfeld JP, Keane WF, Kurokawa K, et al. The losartan renal protection study - Rationale, study design and baseline characteristics of RENAAL (Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan). JRAAS - J Renin-Angiotensin-Aldosterone Syst [Internet]. 2000 Jul 22 [cited 2022 Mar 14];1(4):328–35. Available from: https://journals.sagepub.com/doi/abs/10.3317/jraas.2000.062
4. Carey RM, Siragy HM. The intrarenal renin–angiotensin system and diabetic nephropathy. Trends Endocrinol Metab. 2003 Aug 1;14(6):274–81.
5. Ecelbarger CM, Rash A, Sinha RK, Tiwari S. The effect of chronic candesartan therapy on the metabolic profile and renal tissue cytokine levels in the obese zucker rat. Mediators Inflamm. 2010;2010.

6. Effat Souri, Hassan Jalalizadeh, Shahrooz Saremi. Development and Validation of a Simple and Rapid HPLCMethod for Determination of Pioglitazone in HumanPlasma and its Application to a Pharmacokinetic Study [Internet]. Tehran; 2008 Oct. Available from: https://academic.oup.com/chromsci/article/46/9/809/525442
7. Hanafy A, Mahgoub H. A Validated HPLC Method for the Determination of Linagliptin in Rat Plasma. Application to a Pharmacokinetic Study. J Chromatogr Sci. 2016 Oct;54(9):1573–7.
8. Hollenberg NK, Price DA, Fisher NDL, Lansang MC, Perkins B, Gordon MS, et al. Glomerular hemodynamics and the renin-angiotensin system in patients with type 1 diabetes mellitus. Kidney Int. 2003 Jan 1;63(1):172–8.
9. Jeong H-C, Seo Y-H, Gu N, Rhee MY, Shin K-H. Determination of candesartan or olmesartan in hypertensive patient plasma using UPLC-MS/MS. Transl Clin Pharmacol [Internet]. 2021 Dec 1 [cited 2022 Mar 14];29(4):226–38. Available from: https://doi.org/10.12793/tcp.2021.29.e21
10. Nakamura T, Yamamoto E, Kataoka K, Yamashita T, Tokutomi Y, Dong YF, et al. Beneficial effects of pioglitazone on hypertensive cardiovascular injury are enhanced by combination with candesartan. Hypertension. 2008 Feb;51(2):296–301.
11. Noda M, Matsuo T, Nagano-Tsuge H, Ohta M, Sekiguchi M, Shibouta Y, et al. Involvement of Angiotensin II in Progression of Renal Injury in Rats With Genetic Non-insulin-Dependent Diabetes Mellitus (Wistar Fatty Rats). Jpn J Pharmacol. 2001 Jan 1;85(4):416–22.
12. Resubun, R. M. S., Razak, A., Arifin, A., Indar, I., Malloangi, A., & Thamrin, Y. (2022). The intrinsic and extrinsic motivation on the performance of midwife in community health center. International Journal of Health Sciences, 6(2), 588–596. https://doi.org/10.53730/ijhs.v6n2.7387
13. Shaista Hamid, Anwar Ejaz Beg, Iyad Naeem Muhammad, Sohail Hassan, Amir Hassan, Arfa Akram, et al. Development and validation of HPLC method for the determination of Candesartan in human plasma - PubMed [Internet]. Pak. J. Pharm. Sci. 2018 [cited 2022 Mar 14]. p. .2323-2327. Available from: https://pubmed.ncbi.nlm.nih.gov/30473499/
14. Suryasa, I. W., Rodríguez-Gámez, M., & Koldoris, T. (2021). The COVID-19 pandemic. International Journal of Health Sciences, 5(2), vi-ix. https://doi.org/10.53730/ijhs.v5n2.2937
15. Tight Blood Pressure Control and Risk of Macrovascular and Microvascular Complications in Type 2 Diabetes: UKPDS 38 on JSTOR.
16. Vijay VIkram Singh, Partha Chaudhary, Hema B, Richa Tiwari. Method development of Pioglitazone by UV Spectrophotometer | Insight Medical Publishing [Internet]. Insight Medical Publishing,International Journal ofDrug Development and Research. 2014 [cited 2022 Mar 14]. Available from: https://www.ijddr.in/drug-development/method-development-of-pioglitazone-by-uv-spectrophotometer.php?aid=5715
17. Ware AL, Pekamwar. Development and Validation of Bioanalytical UV-Spectrophotometric Method for Determination of Candesartan and Development and Validation of UV-Spectrophotometric Method for Determination of Candesartan in Bulk Drug and Formulation - ProQuest. . Asian J Pharm Anal [Internet]. 2021 Jun [cited 2022 Mar 14];11(2):79–83. Available from: https://www.proquest.com/docview/2553540484?pq-origsite=gscholar&fromopenview=true

18. Widana, I. K., Sumetri, N. W., & Sutapa, I. K. (2018). Effect of improvement on work attitudes and work environment on decreasing occupational pain. International Journal of Life Sciences, 2(3), 86–97. https://doi.org/10.29332/ijls.v2n3.209
19. Wild S, Roglic G, Green A, Sicree R, King H. Global Prevalence of Diabetes. Diabetes Care. 2004 May;27(5):1047–53.
20. Xavier CM, Basavaiah K. Implementation of Quality by Design for the Development and Validation of Pioglitazone Hydrochloride by RP-UPLC with Application to Formulated Forms. ISRN Chromatogr. 2012 Dec 19;2012:1–11.
21. Yki-Järvinen H. drug therapy Thiazolidinediones.