International Journal of Pharmaceutical Science and Research
ISSN: 2455-4685; Impact Factor: RJIF 5.28
Received: 01-11-2019; Accepted: 03-12-2019; Published: 01-01-2020
www.pharmacyjournal.net
Volume 5; Issue 1; January 2020; Page No. 01-05

A simple, sensitive, high-throughput and robust method for estimation of candesartan in human plasma using LC-MS/MS

A Chaitanya Krishna[1*], Dr. Cheepurupalli Prasad[2], Rajaram S Patil[3]
[1] Research scholar, Faculty of Pharmacy, Pacific Academy of Higher Education and Research University, Udaipur, Rajasthan, India
[2, 3] Associate Professor, Vignan Institute of pharmaceutical Technology, Visakhapatnam, India

Abstract
A simple, sensitive, high-throughput and robust method for estimation of candesartan in human plasma using LC-MS/MS was developed and validated.200 µL plasma samples were acidified and subjected to liquid-liquid extraction with tert-butyl-methyl-ether: dichloromethane (TBME: DCM-60:40). Dried residue is reconstituted and 5 µL of the sample is injected onto LC-MS/MS. Candesartan is separated on ACE C18, 50 x 4.6 mm, 3 µ column at a flow rate of 0.5 mL/min with a total run time of 2.0 mL/min and a single SRM transition (m/z: 441.100→263.080) was used to quantify the analyte from 1-490 ng/mL. Calibration curve standards between 1-490 ng/mL range showed good linearity with a correlation coefficient ($r^2 > 0.993$) in 5 PA batches. % CV for both inter and intra-day was less than 9%. % Nominal for inter and intra-day was between 98.52-105.21. Mean recovery is 62.57 % for candesartan and the IS normalized matrix factor was 1.02.

Keywords: Candesartan, LC-MS/MS, Bio-analytical method, LLE

1. Introduction
Candesartan chemically known as cyclohexyl 1-hydroxyethyl carbonate (Cilexetil acetate) is an angiotensin II receptor antagonist used in the management of hypertension and congestive heart failure [1]. Followed by a single oral dose of 12 mg candesartan, C_{max} of 95.2 ng/ml was observed in healthy subjects without any serious adverse events [2]. Analysis of candesartan in human plasma has to be performed from sub-nanogram levels to quantify the biological samples and there is a requirement to develop a cost-effective, simple, sensitive, high-throughput and robust method to comfortably analyze the samples of all dosage forms by using Liquid chromatography-tandem mass spectrometry (LC-MS/MS). Several LCMS methods are available for estimation of candesartan in human plasma [3-14] with a lower limit of quantitation between 1.0-10.0 ng/mL by using various sample preparation techniques. A detailed review of the literature shows that methods based on protein precipitation [3-5] reported poor sensitivity (1.2 - 5.0 ng/mL). Though very low volumes of denaturating agents are required for precipitation, clean-up of the sample will remain improper. Clogging of the LC tubing and ion-source capillaries increase the frequency of instrument breakdown and thereby the cost of maintenance also. Moreover, during method development, it was understood that the extraction recovery and sensitivity were severely affected due to ion suppression and relative matrix effects. Hence, protein precipitation was not used for extracting the biological samples.
Methods using solid-phase extraction (SPE) [4-10] were also studied. It was observed that the summation of ions was applied for achieving the desired sensitivity levels of 1.0 ng/mL [6]. Either the sample analysis required online solid-phase extraction system [10] or lower particle size columns for better resolution apart from SPE cartridges used for better clean-up of the samples. Hence the methods employing SPE were not cost-effective. The feasibility of liquid-liquid extraction was studied [11-14]. Only one method was found to be more useful for quantifying the entire dose range of candesartan and a linear range of 1-500 ng/mL [13] was achieved only after using Ultra-Performance Liquid Chromatography tandem mass spectrometry (UPLC-MS/MS). Also, the retention time of analyte at a flow rate of 0.8 mL/min on the Phenomenex Gemini NX C18 reverse-phase column (4.6 x 150 mm, 5 µm) was reported to be 0.89 minutes. Though this method uses low (200 µL) sample volume to minimize the matrix effect, candesartan retention time is less than 1 minute on a 150 mm column (elution in void volume) which may result in irregular response when the column is extensively used. Therefore, there are strong chances of response variation. Hence a simple, sensitive, high-throughput and robust method was developed for estimation of candesartan in human plasma using basic HPLC conditions with-out compromising the throughput of the method. The current method has been validated over a range of 1-490 ng/mL. Liquid-liquid extraction has been employed and the separations were achieved on ACE C18, 50 x 4.6 mm, 3µ column with very minimal cost per sample analysis.

2. Materials and methods
Methanol (gradient grade), acetonitrile (gradient grade), acetic acid, ammonium Formate (GR grade) was purchased from Merck. Water (LCMS grade) was used in house from the Milli Q system. Candesartan and candesartan D_4 were procured from Vivan life sciences. Structure of candesartan is presented below in Figure 1.

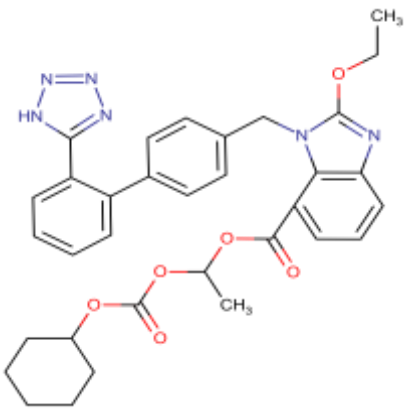


Fig 1: Structure of candesartan

2.1 Instrumentation and analytical conditions

Method has been developed and validated using Shimadzu LC system interfaced with a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc). Heated electrospray-ionization source was operated in the positive mode. Chromatographic separations were performed using ACE C18 (50×4.6 mm inner diameter, 3µ particle size; Thermo Fisher Scientific Inc) at a column temperature of 40℃. 5 mM ammonium formate pH 3.0 (adjusted with formic acid) in water and methanol was used as the mobile phase in pump A and pump B respectively without premixing. Optimization has been performed in isocratic flow conditions with the composition of phase A ranging from 50 to 10 % out of which 10 % phase A and 90 % phase B has been found to give optimal results at a flow rate of 0.5 mL/min. Retention time of candesartan and candesartan D_4 was found to be the same at 0.90 mins with a total run time of 2 minutes.

The following parameters were optimal for candesartan mass spectrometry analysis: Sheath gas 40 (arb), auxiliary gas pressure 20 (arb), capillary temperature 350 °C, Q2 gas pressure 1.5 m Torr, ion spray voltage 4500 V, and vaporizer temperature 300℃. SRM (Selected reaction monitoring) transitions for quantification were m/z 441.100→263.080 for candesartan and 445.100→ 267.140 for candesartan D_4.

2.2 Preparation of standard solutions and quality control samples

Standard solutions of candesartan (100 µg/mL) and candesartan D_4(100 µg/mL) were prepared in methanol. Intermediate stock solutions of both analyte and internal standard (10 µg/mL) were prepared in diluent (50% methanol in water) along with internal standard dilution (5 µg/mL). Nine level calibrators and four-level controls were prepared in human plasma containing dipotassium ethylenediaminetetraacetic acid (K_2EDTA) as an anticoagulant ranging from 1.000-491.250 ng/mL and 1.000-442.125 ng/mL respectively.

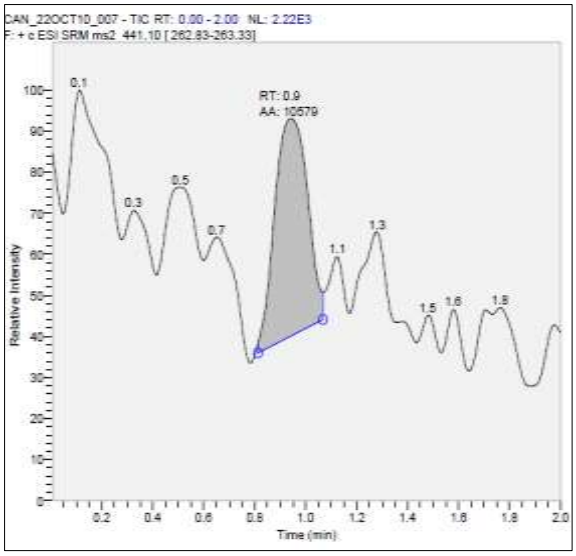
2.3 Sample preparation

10 µL of internal standard was added to 200 µL pre-spiked plasma samples and 10 µL of diluent has been added to the blank samples in prelabelled Radio Immuno Assay (RIA) vials and vortexed to mix well. To the vials add 200 µL of 1.0 acetic acid, vortex to mix and proceed for liquid-liquid extraction. Add 4.0 mL of tert-butyl-methyl-ether: dichloromethane (TBME: DCM-60:40%, V/V) and vortex for 15 minutes. Centrifuge the samples at 3500 rpm at 4 °C for 15 minutes. Transfer 3.6 mL of supernatant to another glass tube and evaporate the solvent under the stream of nitrogen gas until dryness. Reconstitute the residue with 2.0 mL of the mobile phase and load the samples onto prelabelled High-performance liquid chromatography (HPLC) vials. Inject 5 µL samples into the LC-MS/MS system.

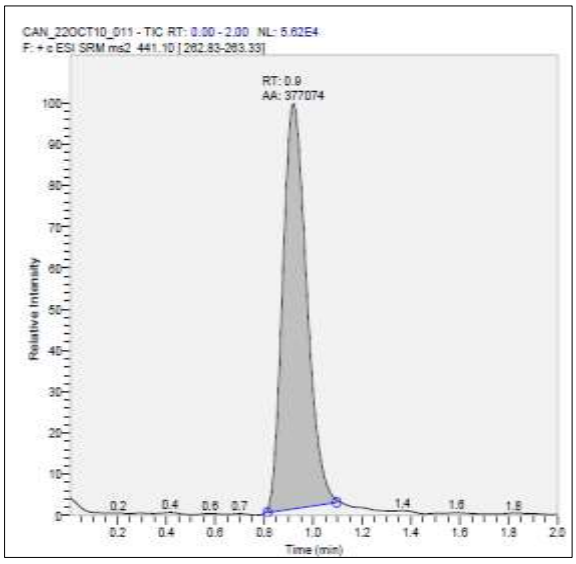
3. Results and discussion

3.1 Method Validation

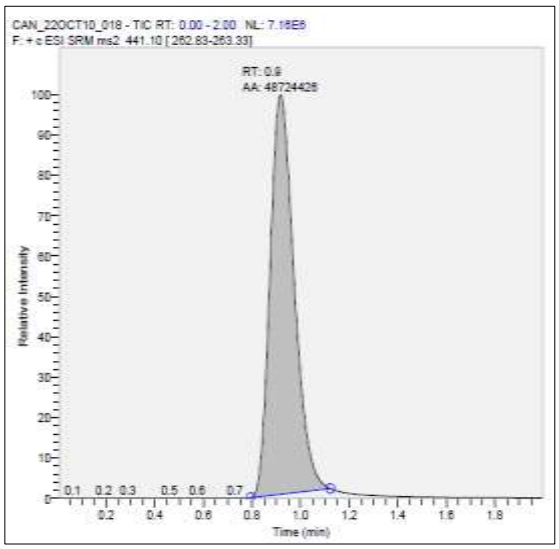
Method has been validated for selectivity, linearity, precision, accuracy, recovery and stabilities as per current Food and drug administration, United states department of health and human services (USFDA) recommendations. Representative chromatograms and a calibration curve of candesartan are shown in Figure 2 and 3:



Extracted Blank



Extracted LLOQ



Extracted ULOQ

Fig 2: Representative chromatograms of Candesartan

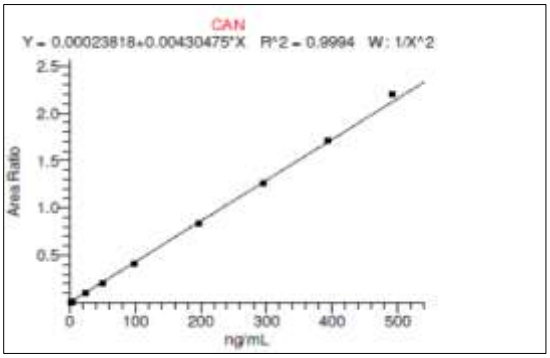


Fig 3: Calibration curve of candesartan in human plasma from 1-490ng/mL

5 Precision and accuracy (P&A) batches (includes ruggedness and stability PA batch) were analyzed with the calibration cure ranging from 1-490 ng/mL. A straight-line equation (y=mx+c) with $1/x^2$ weighing factor has been used to quantify the back-calculated concentration of the calibrators and the coefficient of determination (r^2) was greater than 0.993 in all 5 PA batches.

Specificity and selectivity of the method was assessed in 6 different lots of human plasma containing K_2EDTA as an anticoagulant. Haemolyzed and lipidemic (each lot) were also used for the evaluation of the selectivity of candesartan. % interference in blank was found to be less than 6 % in all lots when compared against the LLOQ area of candesartan.

Intra-day precision and accuracy was evaluated in 6 replicates of control samples at LLOQ QC, LQC, MQC and HQC over one PA batch was found to be between 5.12-8.88 % and 99.16-105.21 respectively.

Inter day precision and accuracy experiments were evaluated in 4 batches at the same levels mentioned above and the results were found to be between 4.68-8.01 % and 98.52-103.03 respectively.

Matrix effect was studied for both candesartan and candesartan D_4 in eight lots of plasma (6 normal, 1 haemolyzed and 1 lipidemic plasma). IS normalized matrix factor was calculated as a ratio of response ratio of post extracted spiked sample upon aqueous sample at both HQC and LQC concentration levels and mean IS normalized matrix factor was found to be 1.02 and 1.00 respectively.

Average recovery of candesartan was obtained by calculating the response ratio of extracted and aqueous samples at LQC, MQC, HQC levels and was found to be 62.57% and 84.95% for candesartan and candesartan D_4 respectively.

Stability experiments in the matrix were conducted for bench-top, freeze-thaw (at -50 °C and at -20 °C), autosampler, dry extract and long-term storage (at -50 °C). Stock solution verification, reinjection reproducibility, ruggedness and dilution integrity were also performed during method validation. Results of these experiments were provided in the method validation summary table (Table 1)

Table 1: Method Validation Summary

Calibration Curve Range		**0.983 to 491.250 ng/mL**
Lower Limit of Quantification		0.983 ng/mL
Limit of Detection /Sensitivity (983 pg/mL)	Accuracy (% Nominal)	116.870
	Precision (%CV)	8.22
Stock Solution Verification	Precision (% CV)	Candesartan = 3.38/1.96
		Candesartan D_4 = 3.43/1.80
Intra-day Precision and Accuracy	Accuracy (% Nominal)	99.16 to 105.21
	Precision (% CV)	5.12 to 8.88
Inter-day Precision and Accuracy	Accuracy (% Nominal)	98.52 to 103.03
	Precision (% CV)	4.68 to 8.01
Ruggedness (Different column)	Accuracy (% Nominal)	101.04 to 105.93
	Precision (% CV)	5.08 to 8.78
Reinjection Reproducibility (15 hrs.30 mins)	Accuracy (% Nominal)	100.73 to 110.54
	Precision (% CV)	1.14 to 5.42
% Recovery	LQC	63.79
	MQC	55.87
	HQC	68.04
	Mean Recovery of candesartan	62.57
	Mean Recovery of candesartan D_4	84.95
Dilution Integrity	Accuracy (% Nominal)	$1/2^{nd}$ dilution factor=98.44
		$1/5^{th}$ dilution factor=98.90
	Precision (% CV)	$1/2^{nd}$ dilution factor =1.29
		$1/5^{th}$ dilution factor = 3.49
IS Normalized Matrix Factor	LQC = 1.005	HQC = 1.020
Bench Top Stability in matrix	Precision (% CV)	Stability LQC = 3.47

(4.30 hrs.)		Stability HQC = 5.18
		Comparison LQC = 2.54
		Comparison HQC = 7.72
	Accuracy (% Nominal)	Stability LQC = 97.74
		Stability HQC = 102.37
		Comparison LQC= 101.21
		Comparison HQC=101.08
Auto Sampler Stability (38 Hrs 00 mins)	Precision (% CV)	Stability LQC = 3.47
		Stability HQC = 5.18
		Comparison LQC = 6.34
		Comparison HQC = 6.76
	Accuracy (% Nominal)	Stability LQC = 97.74
		Stability HQC = 102.37
		Comparison LQC= 89.12
		Comparison HQC= 95.96
Dry Extract Stability (28 hrs 30 mins)	Precision (% CV)	Stability LQC = 3.47
		Stability HQC = 5.18
		Comparison LQC = 2.68
		Comparison HQC = 6.95
	Accuracy (% Nominal)	Stability LQC = 97.74
		Stability HQC = 102.37
		Comparison LQC= 99.28
		Comparison HQC= 102.14
Freeze thaw stability at -50 °C (3rd Cycle)	Precision (% CV)	Stability LQC = 3.47
		Stability HQC = 5.18
		Comparison LQC = 1.92
		Comparison HQC = 1.49
	Accuracy (% Nominal)	Stability LQC = 97.74
		Stability HQC = 102.37
		Comparison LQC= 89.61
		Comparison HQC=90.09

4. Conclusion: The current method has been successfully validated as per USFDA guidelines and all experiments were found to be with-in acceptance criteria. During method development and validation, sample volume as low as 200 µL was used to minimize the matrix effects. The developed method is very cost-effective. The set-up cost required for performing the experiments is also very minimal. Therefore, the cost per sample analysis is very less. Retention time of 0.9 mins on a short column ensures sufficient retention thereby reducing the relative matrix effects. A simple, sensitive, high-throughput and robust method for estimation of candesartan in human plasma using LC-MS/MS has been developed and validated. This method can be used for pharmacokinetic applications.

5. References

1. Drugbank.ca [Internet]. https://www.drugbank.ca/drugs/ DB00796. 18-Dec, 2019.
2. Hoogkamer JF, Kleinbloesem CH, Ouwerkerk M, Högemann A, Nokhodian A, Kirch W, *et al*. Pharmacokinetics and safety of candesartan cilexetil in subjects with normal and impaired liver function, Eur J Clin Pharmacol, 1998; 54(4):341-5.
3. Shailesh T. Prajapati, Pratik K. Patel, Marmik Patel, Vijendra B. Chauhan and Chhaganbhai N. Patel. Development and validation of the liquid chromatography-tandem mass spectrometry method for quantitative estimation of candesartan from human plasma, Pharmaceutical Methods. 2011; 2:130-4.
4. Hong-gang Lou, Zou-Rong Ruan, Bo Jiang. Rapid determination of candesartan in human plasma by liquid chromatography tandem mass spectrometry and its application to a bioequivalence study, Journal of Liquid Chromatography & Related Technologies. 2012; 35(8):1027-37.
5. Kim JR, Kim S, Huh W and Ko JW. No pharmacokinetic interactions between candesartan and amlodipine following multiple oral administrations in healthy subjects, Drug Des Devel Ther. 2018; 12:2475-2483.
6. Vinayender Adireddy, Bhavani Prasanna Kumar Bimireddy, Chalapathi Rao Pulipati and Venkateswarlu Ponneri. A simple and rapid determination of candesartan in human plasma by LC-MS/MS, International Journal of Pharmacy. 2017; 7(4):47-54.
7. Patel B, Jangid AG, Suhagia BN and Desai N. The simultaneous UPLC–MS/MS determination of emerging drug combination; candesartan and chlorthalidone in human plasma and its application, Biomedical Chromatography. 2017; 31(9):1-11.
8. Vanja Forjan, Lea Cvitkovič Maričič, Helena Prosen and Darinka Brodnjak Vončina. Determination of Candesartan in Human Plasma with Liquid Chromatography-Tandem Mass Spectrometry, Acta Chimica Slovenica. 2016; 63:38-46.
9. Vijaya Kumari Karra, Nageswara Rao Pilli, Jaswanth Kumar Inamadugu and J.V.L.N. Seshagiri Rao. Simultaneous determination of pioglitazone and candesartan in human plasma by LC-MS/MS and its application to human pharmacokinetic study, Journal of Pharmaceutical Analysis. 2012; 2(3):167-73.
10. Levi M, Wuerzner G, Ezan E and Pruvost A. Direct analysis of valsartan or candesartan in human plasma and urines by on-line solid phase extraction coupled to electrospray tandem mass spectrometry, Journal of Chromatography B. 2009; 877(10):919-26.
11. Bharathi DV, Hotha KK, Chatki PK, Satyanarayana V and Venkateswarlu V. LC-MS/MS method for simultaneous estimation of candesartan and

hydrochlorothiazide in human plasma and its use in clinical pharmacokinetics, Bioanalysis. 2012; 4(10):1195-204.
12. Bonthu Mohan Gandhi; Atmakuri Lakshmana Rao and Jangala Venkateswara Rao. Simultaneous determination of candesartan and hydrochlorothiazide in human plasma by LC-MS/MS, Braz. J. Pharm. Sci. 2018, 54(1):e17381.
13. Bhupinder Singh, Rama S. Lokhandae, Ashish Dwivedi, Sandeep Sharma, Naveen Dubey. Improved simultaneous quantitation of candesartan and hydrochlorothiazide in human plasma by UPLC–MS/MS and its application in bioequivalence studies, Journal of Pharmaceutical Analysis. 2014; 4(2):144-152.
14. Olga S. Brushinina, Roman V. Gurto, Maksim S. Timofeev, Galina A. Frelikh, Vladimir A. Slepichev, Elena A. Yanovskaya, *et al*. Determination of Candesartan and Hydrochlorothiazide in Human Plasma by HPLC Coupled with Mass Spectrometry, International Journal of Analytical Mass Spectrometry and Chromatography. 2014; 2:25-32.