

# Development and validation of HPLC method for the determination of Candesartan in human plasma

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**Abstract:** Candesartan (CAN), an ARB-blocker, antihypertensive, was analyzed in human plasma by a simple, accurate and precise RP-HPLC (reverse phase-High performance liquid chromatography assay method which was then validated for its accuracy, specificity and precision. The mobile phase has a constitution of acetone, diethylamine and distilled water, while Phosphoric acid was used to adjust the pH to 2.5±0.1. This mobile phase was run at 1.1ml/min and the fluorescence wavelength was set to 392 nm. A C-18 HPLC, column particle size (5 µm) Mediterranean Sea ® L x 1.D. 25cm x 4.6 mm (Supelcosil) , with auto sampler injection volume of 30µl ,an internal standard Valsartan was utilized for chromatographic detection. Candesartan took a retention time of 6±0.5 minutes. This method was validated by the parameters of selectivity, accuracy, precision, repeatability, reproducibility, recovery, linearity and stability. Candesartan's calibration curves were found to be linear in the range of 200ng/ml to 3.125ng/ml and the coefficient of determination ( $r^2$ ) was found to be 0.99. Analytical recovery obtained was above 88%. Hence, this method has been found to be useful for determining Candesartan in plasma.

**Keywords:** Candesartan, HPLC, human plasma, UV-Fluorescence.

## INTRODUCTION

Candesartan (CAN)  $C_{24}H_{20}N_6O_3$ , is highly potent, selective Angiotensin II receptor blocker (ARB). Candesartan cilexetil, the prodrug is hydrolyzed to candesartan after administration by ester hydrolysis and has an estimated bioavailability of 14% (Gleiter *et al.*, 2004; Gleiter & Mörike 2002; Huang *et al.*, 2013; Husain *et al.*, 2011). CAN is used in patients with kidney disease (diabetic nephropathy) and congestive heart failure, to treat arterial hypertension (Joost *et al.*, 2011; Kjeldsen *et al.*, 2010; Kjeldsen *et al.*, 2014; Yusuf *et al.*, 2003). Therapeutic drug monitoring and investigational clinical studies usually benefit from fast, precise, accurate analytical methods to reveal significant pharmacokinetic effects in the body after ingesting candesartan (Gleiter *et al.*, 2004).

The techniques employed are ultraviolet absorption, Liquid chromatography-Mass Spectrometry (LC-MS), HPLC-UV, Electrospray-MS, fluorescence, liquid chromatography tandem mass spectroscopy. (Brushinina *et al.*, 2014; Ferreirós *et al.*, 2007; Karra *et al.*, 2012; Lou *et al.*, 2012; Phechkrajang *et al.*, 2017; Sunandamma, 2013) and the use of MALDI-TOFMS with selected reaction monitoring has been employed to detect CAN in biological fluids (Karra *et al.*, 2012; Nakanishi *et al.*, 2013). The structural formula of candesartan is given in fig. 1 (Kalyani & Deshmukh, 2016).

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## MATERIALS AND METHODS

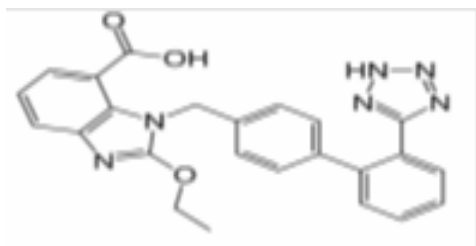
Standard sample of CAN was obtained from Toronto Research Chemicals, C175575. Ortho-phosphoric acid (Merck, Darmstadt, Germany). Valsartan, diethyl amine and acetonitrile HPLC grade and HPLC grade water (Merck). Analysis was performed through HPLC pump, LC-10AT VP with fluorescence detector. All solutions were filtered and ultrasonicated. Plasma was obtained from a healthy volunteer and stored under -80°C and was freeze-thawed and frozen after usage.

### Chromatographic conditions

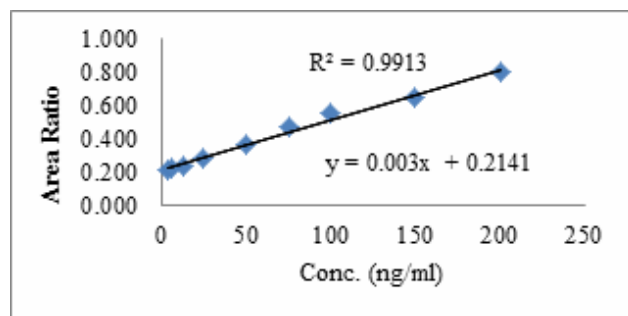
Mobile phase comprised of acetonitrile; water in 1:1 ratio which was adjusted to 0.02M Ortho-phosphoric acid at a flow rate of 1.1ml/min in the column oven. The detection was carried out at 247nm using fluorescence detector at  $E_{m\lambda}=392nm$ , high sensitivity. A C-18 HPLC, column particle size (5µm) Mediterranean Sea ® Lx1.D. 25cmx 4.6mm (Supelcosil) was used. The filtration of mobile phase was done through micron millipore filters. The sample injection volume was 30µl.

### Preparation of mobile phase

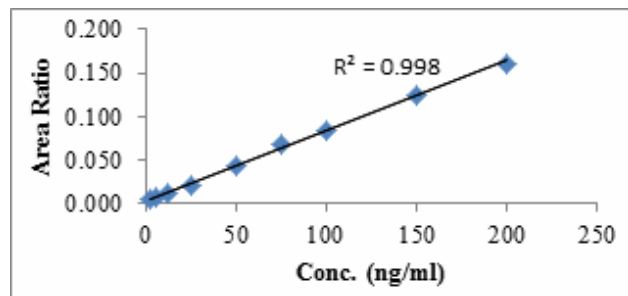
Mobile phase was composed of 0.02M phosphoric acid buffer and acetonitrile, in the ratio of (50:50). Phosphoric acid buffer was made according to the procedure (Bandyopadhyay 2013). The mobile phase pH was adjusted to 3.20±0.2.



**Fig. 1:** chemical structure of Candesartan.



**Fig. 2:** Linearity of Calibrators in Plasma.(Note: Area Ratio expresses the ratio of peak areas of Candesartan compared to Valsartan, the internal standard)



**Fig. 3:** Linearity of Calibrators in Mobile Phase. (Note: Area Ratio expresses the ratio of peak areas of Candesartan compared to Valsartan, the internal standard).

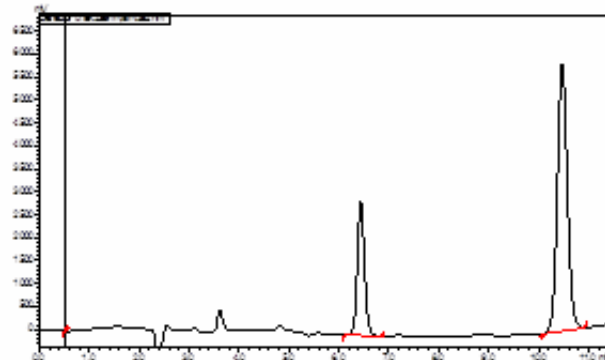
**Preparation of standard solution**

Standard stock solutions of CAN and Valsartan (Internal standard) were prepared by dissolving 10mg of Candesartan in 10ml methanol to obtain a final concentration of 1mg/ml. Further stock solutions were serially diluted to prepare; 10,000ng/ml, 1000ng/ml, 180ng/ml, 90ng/ml and 10ng/ml as the lowest concentration.

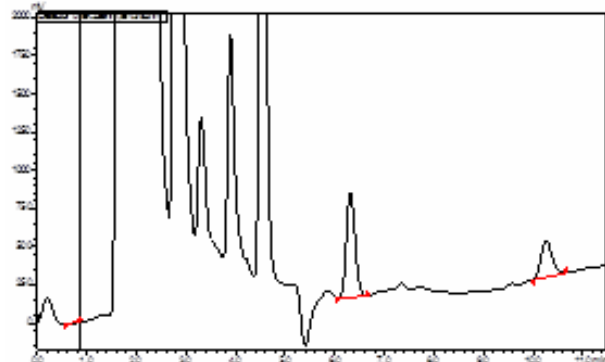
**Preparation of plasma sample solution**

A CAN stock solution of 1000ng/ml was prepared in plasma, serial dilutions were prepared by taking measured volumes from CAN stock and volume made up to 1500µL by fresh plasma in order to get spiked plasma. For the procedure, 300µl of prepared plasma sample solution was taken with 600µL of Acetonitrile, which was added in the ratio 1:2. 20µl of Internal Standard, Valsartan (0.1µg/ml) was also added to spiked plasma. The sample of stock

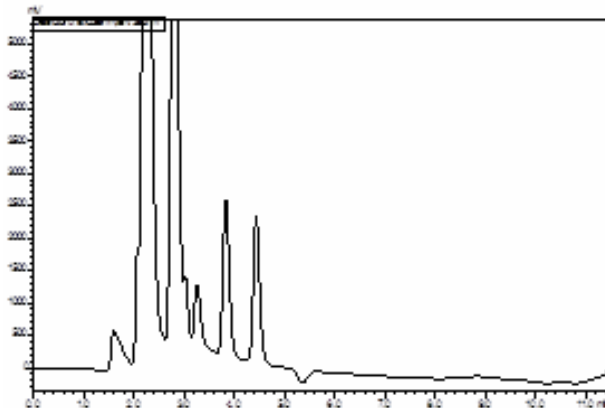
solution was mixed in a Vortex mixer (Whirl mixer, England) for 1 minute and then centrifuged using centrifuge (Heraeus-Osterode Germany) at 10,000rpm for ten minutes. The supernatant was collected and was added to HPLC glass vials. 30µl of the sample was injected by the auto-sampler and the flow rate was 1.1ml/min.



**Fig. 4:** Candesartan and Valsartan (I.S) in Mobile phase



**Fig. 5:** Chromatogram of Candesartan and Valsartan in plasma.



**Fig. 6:** Chromatogram of Blank Plasma

**Calibration curve**

A calibration curve was prepared by taking various concentrations of 3.125ng/ml-200ng/ml of CAN in mobile phase and plasma. The values were then fitted through in a linear equation to determine coefficient of variation (r<sup>2</sup>).

**Table 1:** INTRADAY- Measured Conc. (ng/ml) during different timings of the day over 3 days

Conc. (ng/ml) N= 5	Mean Conc. (ng/ml) $\pm$ SD			Mean Accuracy% and %CV		
	Day 1	Day2	Day3	Day 1	Day2	Day3
3.125	3.06 $\pm$ 0.27	3.27 $\pm$ 0.29	3.29 $\pm$ 0.34	97.86 $\pm$ 8.76	104.74 $\pm$ 8.93	100 $\pm$ 10.34
10	10.54 $\pm$ 0.39	10.15 $\pm$ 0.82	10.9 $\pm$ 1.08	105.42 $\pm$ 3.66	101.50 $\pm$ 8.06	100.92 $\pm$ 10.68
90	100.20 $\pm$ 2.29	95.85 $\pm$ 6.68	98.96 $\pm$ 1.23	100.20 $\pm$ 2.29	106.50 $\pm$ 6.97	109.26 $\pm$ 1.24
180	178.09 $\pm$ 12.41	175.88 $\pm$ 6.39	190.27 $\pm$ 6.19	178.09 $\pm$ 6.97	97.71 $\pm$ 3.63	105.70 $\pm$ 3.25

**Table 2:** Long term stability of candesartan in plasma

Samples	Low Concentration (10ng/ml)		Middle Concentration (90ng/ml)		High Concentration (180ng/ml)	
	Fresh plasma	4 weeks	Fresh plasma	4 weeks	Fresh plasma	4 weeks
Mean n=3	10.302	10.325	96.056	97.795	176.426	192.504
SD	0.387	0.519	5.934	1.828	6.791	6.267
%CV	0.0460	0.0615	0.0229	0.0751	0.0471	0.0398

**Table 3:** Absolute analytical recovery

Conc. (ng/ml)	Recovery Day 1 (%)	Recovery Day2 (%)	Recovery Day3 (%)
10ng/ml	93.503	91.65	87.31
90ng/ml	90.559	93.60	92.24
180ng/ml	85.814	91.58	86.22
Mean Recovery (%)	89.95	92.28	88.59

**Table 1:** INTERDAY- Measured Conc. (ng/ml) over 3 days.

		Mean Accuracy (%)			
		Actual Conc. (ng/ml)			
		10	90	180	3.125
Calculated Conc.	Day 1	10.54	100.20	181.83	3.15
	Day 2	10.15	95.85	175.88	3.27
	Day 3	10.09	98.96	190.27	3.29
Mean		10.26	98.34	182.66	3.24
Mean accuracy		102.61	109.26	101.48	103.70
		Mean Precision (%CV)			
		Actual Conc. (ng/ml)			
		10	90	180	3.125
Calculated Precision	Day 1	3.66	2.29	6.83	11.43
	Day 2	8.06	6.97	3.63	8.93
	Day 3	10.68	1.40	3.56	11.49
Mean precision		7.47	3.55	4.67	10.61

**Precision and accuracy**

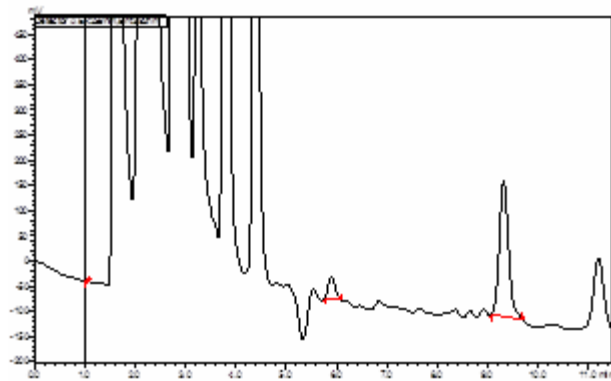
This method's precision (RSD) was found within the acceptable limit of  $\pm 15\%$ . (CF, 2001) Similarly, the accuracy of this analytical method was observed to remain within  $\pm 15\%$  of the actual values. For interday precision, triplicate samples were injected, of the following concentrations; i.e. 10ng/ml, 90ng/ml, 180ng/ml and followed on three consecutive days while for intraday precision, five samples of each concentration were observed. Fresh samples were prepared before analysis.

**Selectivity**

The selectivity for this method was assessed, using ten blank plasma samples, along with detection of the internal standard, Valsartan.

**Limit of detection**

This is the lowest detectable concentration of the analyte within a sample, further expressed as the amount that can be reliably differentiated from the signal ratio obtained within blank samples. For the limit of detection a signal to noise ratio of 3:1 or 2:1 is considered acceptable (CF, 2001).



**Fig. 4:** Lower limit of quantification, LLOQ (3.125ng/ml) in plasma, signified by arrow.

#### **Stability of plasma samples**

To determine the short term and long term stability of CAN in solvent at ambient and refrigeration temperature, four sets of low (10ng/ml), medium (90ng/ml) and high (180ng/ml) concentrations of the spiked calibration standards in plasma were divided in to 18 test tubes. One set of spiked samples was assayed immediately and was considered as standard (100%). The second set was stored at a temperature of  $-4^{\circ}\text{C}$  in a refrigerator for 24 hours and three day (for freeze-thaw stability testing. The third set was stored at  $-20^{\circ}\text{C}$  in a freezer for four weeks, with sampling done after every week. Stability tests were repeated for 4 weeks to detect degradation of standard quality control samples. The results were evaluated and compared these measurements with those of standards and expressed as percentage deviation, percentage mean and recovery. The results are summarized in table 2. The plasma samples were found to be stable for over one week at  $-20^{\circ}\text{C}$  (deep freeze) and 24 hours at room temperature and at  $-4^{\circ}\text{C}$  (refrigerator). For short term-stability, the stability of analyte stock solution was tested for over 3 days in the same assay. The results indicated stability of stock solution for over 3 days at both room temperature and refrigerator temperature. The results are presented in table 1.

#### **LLOQ (Lower limit of quantification)**

Lower limit of quantification (LLOQ) and lower limit of detection met the acceptance criteria of accuracy and precision of  $\pm 15\%$  bias and C.V. from five or more determinations. (CF. 2001). The concentration at which LLOQ can be determined is 3.125ng/ml.

#### **Recovery**

The recovery of an analyte is the response obtained from the detector from an amount of the analyte added to and extracted from the biological matrix, a thorough comparison is made of this plasma sample from the analyte in solvent. Recovery assessment was done using triplicate injections of spiked plasma at the concentrations; 10ng/ml, 90ng/ml, 180ng/ml, respectively. They were analyzed and compared with

respective concentrations for standard solutions, for three days. The data is summarized in table IV.

## **RESULTS**

This procedure for validating Candesartan was according to FDA, CDER guidelines set for bioanalytical method validation. This suitably fits the guidelines for accuracy, precision, selectivity, recovery, sensitivity and stability. The coefficient of variation for the concentration range of 200ng/ml to 3.125ng/ml was found to be  $r^2$  0.991 in plasma and  $r^2$  0.998 mobile phase (fig. 2). The analytical recovery of this method was done on three concentrations; 10ng/ml, 90ng/ml and 180ng/ml. Recovery results were reproducible, consistent and lied within the acceptable range 89.95% -92.28% (table 3) (C, 2001). The interday mean accuracy and precision was determined for 3 days and found to be, 102.61%, 109.26% and 101.48% for days 1, 2, 3, respectively. Similarly, percentage recovery of these three solutions were in the range of 89.69% and 105.80%, after 3 to 4 weeks of appropriate storage conditions ( $-20^{\circ}\text{C}$ ). This indicates the fact that this method resulted in stability limits of  $\pm 15\%$ . Selectivity is the absence of any interference in detecting the analyte. The method was found to be detecting to the LLOQ of 3.125ng/ml (fig. 4). Precision and accuracy were satisfactory i.e. within  $\pm 15\%$  range. The stability of analyte was found to be good for the concentrations 10ng/ml, 90ng/ml and 180ng/ml.

## **DISCUSSION**

Various methods for the detection of CAN have been reported. (Bandyopadhyay S, june 2013) (Ferreirós N, 2007). The current method was modified in terms of pretreatment procedure of biological samples and LLOQ which is essential for obtaining a comprehensive pharmacokinetic profile. The LLOQ of CAN was determined to be 3.125ng/ml (fig. 4), which is less than 5ng/ml, 10ng/ml as reported in previous methods (Karraa, 2012) (Brushinina, 2014). Figs. 2-5 show the calibration curves of candesartan in mobile phase and plasma. The fig. 6 shows, the retention time of endogenous material in blank plasma. This indicates that peak of CAN and Valsartan (IS) obtained at  $6\pm 0.5$  min and 10.4 min are not overlapping plasma peaks which are obtained in the range of  $1-5\pm 0.5$  minutes. The regression equation of the calibration curve was as follows;  $y = 0.003x + 0.2141$ ,  $r > 0.9913$ , where y denotes the peak of area ratio and candesartan plasma concentration. This bioanalytical method gave us a retention time of  $6\pm 0.5$  minutes, which is an improvement compared to previously reported retention time of 6.39 minutes (Khaire, 2016). A mobile phase comprising of phosphoric acid buffer: Acetonitrile in the ratio of 50:50 was found to be optimized, by doing this, plasma peaks obtained were symmetrical and clearly defined. Satisfactory separation between endogenous plasma compounds and drug was obtained. Method

validation was performed as per FDA bioanalytical method validation guidelines (CF, 2001). Linearity of an analytical method should be established within a range which is intended to cover the maximum concentration versus time graph in a pharmacokinetic or bioequivalence study. This proposed method showed good linearity in dynamic range of 3.125ng/ml- 200ng/ml compared to the linearity range 10ng/ml - 250ng/ml (Brushinina *et al.*, 2014). Linearity, recovery, precision and accuracy were statistically confirmed using this method. The findings of the validation experiments were in concordance with previously reported results (Kalyani, 2016).

## CONCLUSION

The developed and validated reverse-phase high performance liquid chromatography method with fluorescence detector for the quantification of candesartan in human plasma was found to be an accurate, simple, less time-consuming, precise and sensitive method is an accurate, with excellent linearity. Therefore, this method can be adopted for the analysis of CAN in plasma for pharmacokinetic and bioequivalence studies.

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