International Journal of Pharmacy
Journal Homepage: http://www.pharmascholars.com

**Original Article** **CODEN: IJPNL6**

# A simple and rapid determination of candesartan in human plasma by LC-MS/MS

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***Received on: 01-08-2017; Revised on: 26-09-2017; Accepted on: 28-09-2017***

**ABSTRACT**

The authors described a novel liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the determination of candesartan in human plasma. The method employs isotope labelled compound, candesartan d4 as internal standard (IS). A simple and one step solid phase extraction (SPE), was used to extract the analyte and the IS. An isocratic mobile phase composed of methanol–5mM ammonium acetate (70:30, v/v) was used to separate the components on $C_{18}$ column. The method was validated in the range of 1.03–307.92 ng/mL as per the US FDA guidelines. Precision and accuracy results were calculated using five successful calibration curves. All stability tests were well within the acceptable limits. A total run time was set at 2.5 min, which allow us to analyze more number of samples in a single run.

***Keywords:*** Candesartan; Solid–phase extraction (SPE); LC–MS/MS; Method validation.

## INTRODUCTION

Candesartan is selective an angiotensin II receptor antagonist used for the treatment of hypertension. Candesartan binds selectively and non-competitively to the angiotensin II receptor type 1, therefore preventing the actions of angiotensin II. The drug finds most significant clinical use in the treatment of hypertension of all grades [1, 2].

A review of literature reveals that few LC-MS/MS methods [3–6] have been reported for determination of candesartan in human plasma. These methods are having limitations like' complicated and expensive extraction procedures or long chromatographic run time. The author Levi *et al.,* 2009 [3] reported a method for the quantification of candesartan in human plasma utilizes on-line sample preparation technique, which is expensive equipment involving many stringent method development protocols. Another method reported by Bharathi *et al.,* 2012 [4] for determination of candesartan in human plasma is more sensitive but a time–cost sample preparation involving liquid–liquid (L–L) extract, evaporation, drying and reconstitution was used in this method for sample preparation.

Another author, Prajapati *et al.,* 2011 [5] published a LC-MS method for the quantification of candesartan in human plasma with an LLOQ of 1.2 ng/mL. The

method utilizes protein precipitation (PP) and not efficient to remove the endogenous compounds such as lipids, fatty acids, and phospholipids completely which is most likely to cause ion suppression. A promising method was reported by Karra *et al.,* 2012 [6] for the simultaneous determination of candesartan and pioglitazone in human plasma. This method utilizes 250 µL of human plasma and the LLOQ was set at 5 ng/mL. The run time was 2.5 min. The analytical method should satisfy the scientists in terms of simplicity, sensitivity, runtime, sample volume, time consumption and efficient extraction procedure [7, 8].

The aim of the present study was to develop and validate a simple, rapid and sensitive LC-MS/MS method for the determination of candesartan in human plasma using isotope labeled compound candesartan d4 as internal standard. The analyte and the IS were extracted from human plasma using one step solid–phase extraction (SPE). In the present method we achieved higher sensitivity (5 folds) with lower plasma volume (50 µL) when compared with earlier reports [6].

## EXPERIMENTAL

Standards and reagents: Reference sample of candesartan (98.04%) and candesartan d4 (98.03%) were obtained from Clearsynth Labs Limited (Mumbai, India). Ammonium acetate and formic acid were purchased from Merck Ltd (Mumbai, India). Ultra-pure methanol was obtained from .T. Baker (Phillipsburg, USA). HPLC grade water was used for the analysis and obtained from Rankem Limited (Mumbai, India). Blank human plasma samples were obtained from Deccan's Pathological Lab's (Hyderabad, India).

LC–MS/MS instrument and conditions: An API–4000 triple quadrupole spectrometer (MDS Sciex, Foster City, CA, USA) coupled with HPLC system (Shimadzu, Kyoto, Japan) was used for the study. The prepared samples were injected (10 µL) on to a Zorbax Eclipse XDB-$C_{18}$ 4.6 x 150 mm, 5 µm) column. An isocratic mobile phase composed of methanol–5mM ammonium acetate (75:25, v/v) was used and delivered at a flow rate of 0.9 mL/min. The electro spray ionization (ESI) source temperature was maintained at 500 °C and voltage was set at 5500 V. The compound dependent parameters viz. the declustering potential (DP), collision energy (CE), collision cell exit potential (CXP) and entrance potential (EP) were 53, 17, 11 and 10 V for candesartan and for the IS. The source parameters viz. the nebulizer gas (GS1), auxiliary gas (GS2), curtain gas and collision gas were set at 40, 40, 25, and 6 psi, respectively. Detection of the ions was carried out in the multiple–reaction monitoring mode (MRM), by monitoring the transition pairs of *m/z* 441.1 precursor ion to the *m/z* 262.9 for candesartan and *m/z* 445.0 precursor ion to the *m/z* 267.1 product ion for the IS. Quadrupoles Q1 and Q3 were set on unit resolution. The chromatographic data was processed by Analyst Software™ (version 1.4.2).

Preparation of stock and working solutions: Candesartan and Candesartan d4 stock solutions were prepared in methanol. Two separate stock solutions were prepared for candesartan and used for the preparation of calibration curve (CC samples) standards and quality control (QC) samples, respectively. All the working solutions were prepared in a mixture of acetonitrile and water (50:50, v/v; diluent).

Calibration standards were prepared in plasma as 1.04, 2.07, 5.18, 10.37, 30.95, 61.89, 123.78, 184.75, 264.34 and 307.92 ng/mL. Equally, quality control (QC) samples were also prepared at concentrations of 1.06 (lower limit of quantitation, LLOQ), 3.15 (low quality control, LQC), 31.85 (medium quality control, MQC1), 141.54 (MQC2) and 252.75 ng/mL (high quality control, HQC) as a single batch at each. All the bulk spiked samples were stored in deep freezer at –70±10 °C.

Sample processing protocol: An aliquot of 50 µL of thawed human plasma sample was mixed with 10 µL of the internal standard working solution (2140 ng/mL of candesartan d4). To this, 50 µL of 2% formic acid solution was added after vortex mixing for 10 s. The sample mixture was loaded onto a Strata-X 33 µm polymeric sorbent cartridge (30 mg/1 mL) that was pre–conditioned with 1.0 mL of methanol followed by 1.0 mL of water. The extraction cartridge was washed with 1.0 mL of 2% formic acid followed by 1.0 mL of water (1 mL each time). Analyte and the IS were eluted with 0.5 mL of mobile phase. Aliquot of 10 µL of the extract was injected into the LC-MS/MS system.

Method validation parameters: The present method was validated as per the recent US FDA guidelines [9]. The parameters determined were carryover test, selectivity, matrix effect, sensitivity, linearity, precision and accuracy, recovery, dilution integrity, and stability.

## RESULTS AND DISCUSSION

Method development: At the initials stage of work mass parameters were tuned in positive and negative

ion modes using ESI source. The MRM technique provides inherent selectivity and sensitivity and hence used for the present study [10, 11]. We observed more response in positive than the negative ion mode. The compound and source dependent parameters were suitable altered to get good and reproducible response. Protonated form of analyte and IS, $[M+H]^+$ ion was the parent ion in the $Q_1$ spectrum and was used as the precursor ion to obtain $Q_3$ product ion spectra. The most sensitive mass transition was observed from m/z 441.1 to 262.9 for candesartan and from m/z 445.0 to 267.1 for the IS. The dwell time for each transition was 200 ms.

Many options were evaluated to optimize the chromatographic conditions. Acetonitrile and methanol in combination with volatile buffers like ammonium acetate and ammonium formate and also acidic buffer like acetic acid and formic acid. The best chromatographic results were achieved with a combination of methanol–5mM ammonium acetate (70:30, v/v) as the mobile phase. The peak shape was good and response was reproducible with Zorbax Eclipse XDB-$C_{18}$ 4.6 x 150 mm, 5 µm) column even at low concentration level. The retention time of analyte and the IS were low enough (1.5 min) allowing a short run time of 2.5 min.

The earlier authors employed LLE [4], PP [5] and SPE [6] to extract the candesartan from biological samples. In the present method we used SPE technique for sample preparation and employed more or less similar conditions as Karra *et al.*, 2012 [6] used. Good and reproducible recoveries were obtained with Strata-X 33 µm polymeric sorbent cartridge (30 mg/1 mL) when compared with other SPE cartridges like HLB and Orpheus $C_{18}$ cartridges. The present method gives highest recovery (>90%) with good and acceptable peak shape. Formic acid (2%) buffer was added to the plasma samples an extraction additive and helped in achieving high recovery for the analyte and the IS. Now regulatory agencies are insisting to use the isotope–labeled drugs as internal standards where matrix effect is possible. These compound will helps us to obtain increase precision and accuracy with no variation in recovery between analyte and the IS. Hence, we used Candesartan d4 as internal standard and found to be best for the present purpose.

Carryover and sensitivity: Carryover experiment was conducted to evaluate the possible carryover in the subsequent runs after injecting the highest concentration of the analyte. Results divulge that there was no significant carryover effect in the blank samples after injection of high concentration of the analyte. The lowest concentration (LOQ) which can measure with acceptable precision and accuracy was set at 1.04 ng/mL. The precision and accuracy of analyte at LLOQ level was found to be 4.05 and 103.18%, respectively.

Selectivity and chromatography: A total of six plasma lots (4 were normal and one lipemic and one haemolyzed) were screened for selectivity test and all lots were found to be free from interference derived from endogenous components (Fig 1). Also, no interference at the retention time of analyte when internal standard was added (Fig 2). Fig. 3 depicts a representative ion–chromatogram for the LLOQ (CS–1) sample (1.04 ng/mL).

Matrix factor: The matrix effect was evaluated by calculating the matrix factor (MF) at LQC and HQC levels. The mean area response of post–extraction spiked samples were compared with mean area of aqueous samples (neat samples) prepared in mobile phase.
IS–normalized MF was calculated using the below formula:

$$\text{IS normalized matrix Factor} = \frac{\text{Peak response area ratio in presence of matrix ions}}{\text{Mean peak response area ratio in absence of matrix ions}}$$

The %CV for IS normalized matrix factor at LQC and HQC level was found to be 2.67% and 0.42%, respectively and IS normalized factor was 1.02 for LQC and 1.00for HQC. The results show negligible matrix effect in all the plasma lots used for the study.
Linearity, precision and accuracy: A total of five successful calibration curves were generated over the concentration range of 1.04–307.92 ng/mL for candesartan. The mean correlation coefficient of the weighted calibration curves generated during the validation was ≥ 0.99. After comparing the two weighting models (1/x and $1/x^2$), a regression equation with a weighting factor of $1/x^2$ of the drug to the IS concentration was found to produce the best fit for the concentration–detector response relationship.

The results for intra–day and inter–day precision and accuracy in plasma quality control samples are presented in Table 1. The results revealed good precision and accuracy. The precision deviation values were all within 15% of the relative standard deviation (RSD) at all QC level, whereas within 20% at LLOQ QCs level. The accuracy deviation values were all within 100 ± 15% of the actual values at all QC level, whereas within 100± 20% at LLOQ QCs level. Stability studies: The analyte and the IS was stable in processed as well as in plasma samples. The processed

samples stability like autosampler stability (41 h), wet extract stability at 2–8 °C (46 h) and reinjection stability (35 h) and analyte stability in plasma samples namely bench top stability (10 h), repeated freeze–thaw cycles (4 cycles) and long term stability at –70 °C for 75 days, the mean % nominal values of the analyte were found to be within ±15% of the predicted concentrations for the analyte at their LQC and HQC levels (Table 2). All the stability results were found to be within the acceptable limits during the entire validation.

Recovery and dilution integrity: The recovery of candesartan was determined at LQC, MQC2 and HQC levels, whereas for the IS was determined at 2140 ng/mL. The mean overall recovery of candesartan was 90.20±2.52% with the precision range of 0.76–7.89%. Similarly, the recovery of IS was 89.69% with the precision range of 9.49–12.18%.

The concentration of unknown samples obtained above the ULOQ can be analyzed by performing the dilution integrity or sample dilution. The ULOQ limit can be extended to 515.61 ng/mL for by 1/2 and 1/4 dilutions with screened human blank plasma. The precision (%CV) for dilution integrity of two-fold and four-fold dilution was found to be 5.95% to 2.79%, while the accuracy results were found to be 103.58% and 102.03%, respectively.

## CONCLUSIONS

The proposed method is simple, rapid and sensitive for the determination of candesartan in human plasma and is validated as per US FDA guidelines. Deuterated internal standard candesartan d4 was used as internal standard to obtain better precision and accuracy results. The method utilizes very low plasma volume (50 µL) for the sample preparation by a simple SPE technique without drying, evaporation and reconstitution steps. Thereby significantly reduces the sample processing time. Also, the run time per sample analysis is 2.5 min which allows analysis of more samples in a single day. From the results of all the validation parameters, we can conclude that the developed method can be useful for bioavailability and bioequivalence (BA/BE) studies and routine therapeutic drug monitoring with the desired precision and accuracy.

## ACKNOWLEDGEMENTS

The authors gratefully acknowledge PCR Laboratories (Hyderabad, India) for providing necessary facilities to carry out this work.

**Table 1: Precision and accuracy data for candesartan**

| Quality control Run | Concentration found Mean±SD (ng/mL) | Precision (%) | Accuracy (%) |
|---|---|---|---|
| **Intra–day variations (*n*=12 at each concentration)** | | | |
| **LLOQ** | **1.09 ± 0.08** | **7.70** | **102.89** |
| **LQC** | **3.44 ± 0.06** | **1.63** | **109.03** |
| **MQC1** | **33.67 ± 0.49** | **1.46** | **105.71** |
| **MQC2** | **151.93 ± 2.42** | **1.59** | **107.34** |
| **HQC** | **261.95 ± 3.33** | **1.27** | **103.64** |
| **Inter–day variations (*n*=30 at each concentration)** | | | |
| **LLOQ** | **1.11± 0.08** | **6.98** | **104.56** |
| **LQC** | **3.31 ± 0.13** | **3.86** | **104.99** |
| **MQC1** | **32.79 ± 1.35** | **4.11** | **102.96** |
| **MQC2** | **147.37 ± 5.07** | **3.44** | **104.12** |
| **HQC** | **256.29 ± 8.31** | **3.24** | **101.40** |
| **Nominal concentrations of LLOQ, LQC, MQC1, MQC2 and HQC are 1.06, 3.15, 31.85, 141.54 and 252.75 ng/mL, respectively.** | | | |

**Table 2: Stability data for candesartan in plasma (*n*=6)**

| Stability test | QC (spiked concentration (ng/mL) | Mean ± SD (ng/mL) | Precision (%) | Accuracy/ Stability (%) |
|---|---|---|---|---|
| **Process[a]** | **3.15** | **3.33 ± 0.18** | **5.28** | **105.69** |
| | **252.75** | **249.18 ± 20.42** | **8.20** | **98.59** |
| **Process[b]** | **3.15** | **3.31 ± 0.35** | **10.65** | **105.10** |
| | **252.75** | **258.51 ± 1.61** | **0.62** | **102.28** |
| **Bench top[c]** | **3.15** | **3.34 ± 0.13** | **3.76** | **106.00** |
| | **252.75** | **259.47 ± 7.88** | **7.88** | **102.66** |
| **FT[d]** | **3.15** | **3.35 ± 0.15** | **4.46** | **106.20** |
| | **252.75** | **253.78 ± 11.96** | **4.71** | **100.41** |
| **Reinjection[e]** | **3.15** | **3.45 ± 0.11** | **3.11** | **109.50** |
| | **252.75** | **272.91 ± 8.24** | **3.02** | **107.97** |
| **Long–term[f]** | **3.15** | **3.36 ± 0.06** | **1.89** | **106.59** |
| | **252.75** | **258.86 ± 1.36** | **0.52** | **102.42** |

**[a] after 41h in autosampler at 10°C; [b] after 46 h at 2–8°C; [c] after 10 h at room temperature; [d] after 4 freeze and thaw cycles; [e] after 35 h of Reinjection;**
**[f] at –70°C for 75 days**

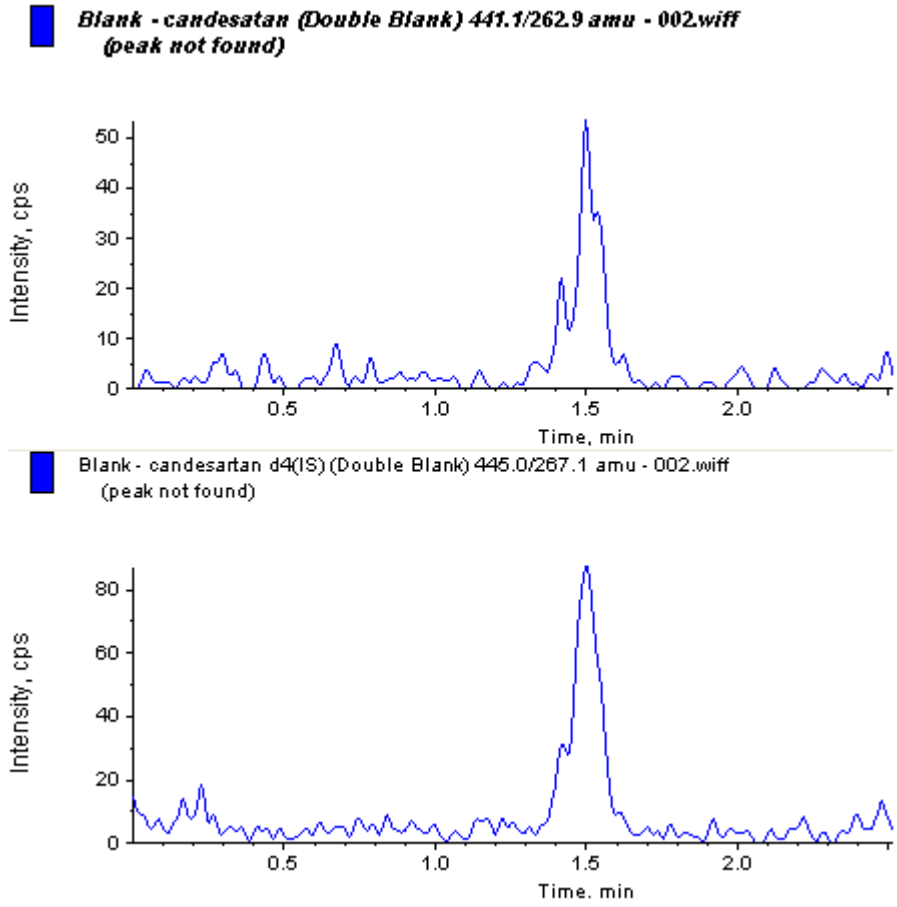


**Figure 1.**
**Typical MRM chromatograms of candesartan (upper panel) and IS (lower panel) in human blank plasma.**

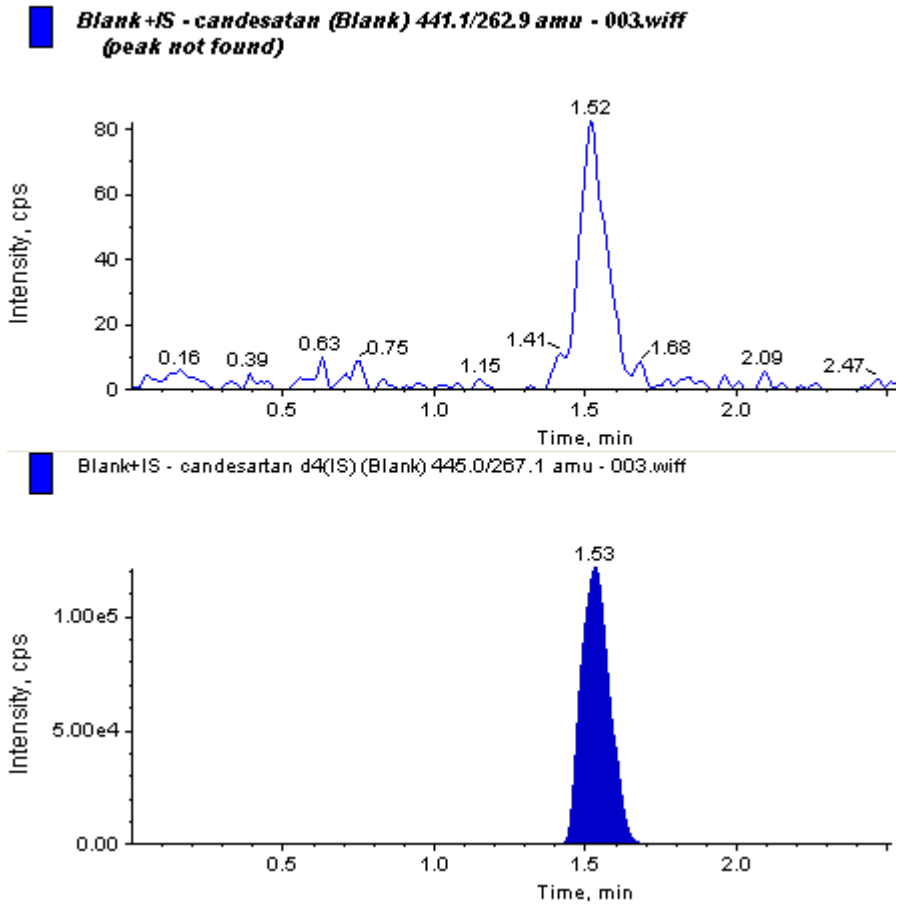


**Figure 2.**
**Typical MRM chromatograms of candesartan (upper panel) and IS (lower panel) in human blank plasma with the IS.**

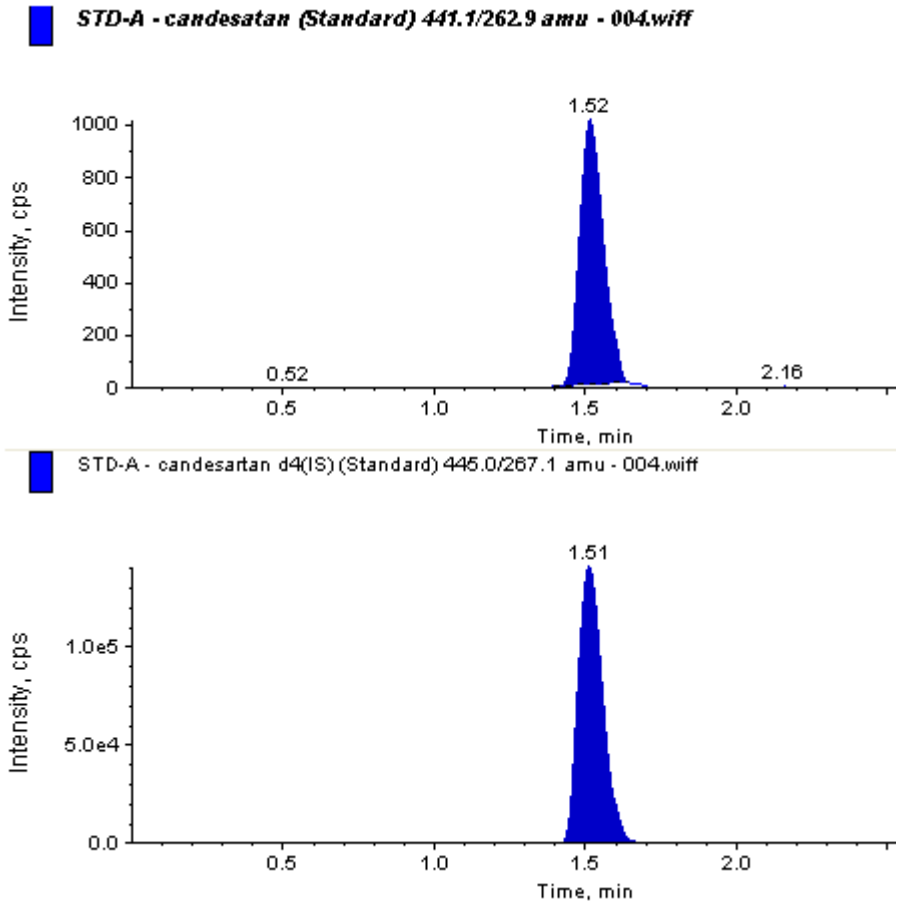


**Figure 3.**
**Typical MRM chromatograms of candesartan (upper panel) and IS (lower panel) in LLOQ sample.**

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