Original Article

Development and validation of the liquid chromatography-tandem mass spectrometry method for quantitative estimation of candesartan from human plasma

Abstract

Introduction: A simple and sensitive liquid chromatography-tandem mass spectrometry method was developed and validated for estimation of candesartan in human plasma using the protein precipitation technique. **Materials and Methods:** The chromatographic separation was performed on reverse phase using a Betasil C_8 (100 x 2.1 mm) 5-µm column, mobile phase of methanol:ammonium tri-floro acetate buffer with formic acid (60:40 v/v) and flow rate of 0.45 ml/min. The protonated analyte was quantitated in positive ionization by multiple reaction monitoring with a mass spectrometer. The mass transitions *m*/*z* 441.2 → 263.2 and 260.2 → 116.1 were used to measure candesartan by using propranolol as an internal standard. **Results**: The linearity of the developed method was achieved in the range of 1.2–1030 ng/ml ($r^2 \geq 0.9996$) for candesartan. **Conclusion**: The developed method is simple, rapid, accurate, cost-effective and specific; hence, it can be applied for routine analysis in pharmaceutical industries.

Key words: Candesaminan, LC-MS/MS method, propranolol

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INTRODUCTION

Candesartan is used in the management of hypertension, and has been investigated in heart failure.[1,2] Candesartan is an anti-hypertensive drug from a category of angiotensin-II receptor antagonists. Angiotensin II is formed from angiotensin I in a reaction catalyzed by angiotensin-converting enzyme (ACE, kinase II). Angiotensin II is the principal pressor agent of the renin–angiotensin system, with effects that include vasoconstriction, stimulation of synthesis and release of aldosterone, cardiac stimulation and renal reabsorption of sodium. Candesartan blocks the vasoconstrictor and aldosterone-secreting effects of angiotensin II by selectively blocking the binding of angiotensin II to the AT1 receptor in many tissues, such as vascular smooth muscle and adrenal gland. Its action is, therefore, independent of the pathways for angiotensin II synthesis.

The chemical name of candesartan is {(±)-1-Hydroxyethyl 2-ethoxy-1-[*p*-(*o*-1*H*-tetrazol-5-ylphenyl) benzyl]-7-benzimidazole carboxylate}.[2] The structure of candesartan is shown in Figure 1. On a detailed literature survey, it was found that there was only one liquid chromatography-tandem mass spectrometry (LC-MS/MS) gradient method reported for the estimation of candesartan from human plasma,[3] and one method was reported to estimate candesartan in rat plasma by LC-MS/MS.[4] Some methods were reported to estimate candesartan from human plasma and solid dosage forms by the high-performance liquid chromatography (HPLC)[5-9] and UV spectrophotometric methods,[10] which were found to be time-consuming and costly. Hence, the objective of the present work was to develop a simple bioanalytical method to estimate candasartan from human plasma with due consideration of accuracy, sensitivity, rapidity, economy,

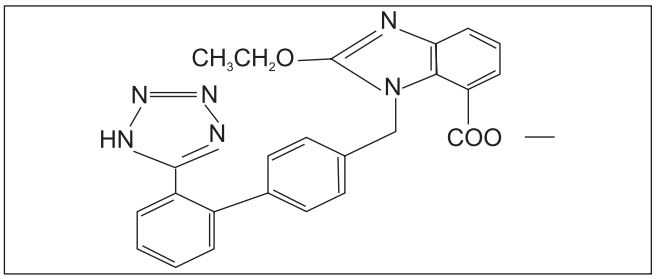

Figure 1: Structure of candesartan

selectivity and stability indicating according to the US-FDA guidelines.

MATERIALS AND METHODS

Chemical and reagents

The working standard or Candesartan and Propranolol as internal standard were gifted by Zydus Cadila Healthcare Limited, Ahmedabad, India. Human plasma samples were procured from Prathama Blood Bank, Ahmedabad, India. Methanol (HPLC grade) and ammonium trifloroacetate (GR grade) were purchased from Spectrochem, Hyderabad, India. Formic acid supra pure grade was purchased from Merck, Mumbai (India) and Milli-Q water was procured from Zydus Cadila Healthcare Limited.

Instrumentation

An HPLC (Shimadzu Corporation, Kyoto, Japan) coupled to an API 4000 mass spectrometer (Thermo Finnigan Ltd., Stafford Ho, UK) was employed for the analysis. A pH meter (Thermo Orion, Asheville, NC , USA , Model 420) and sonicator (Oscar Ultra Sonics, Andheri (E), Mumbai , India OU-72 SPL) were used for this work. The chromatographic conditions were as follows:

Column: Betasil C_8 (100 x 2.1 mm), 5 μm; injection volume: 5 μL; flow rate: 0.45 ml/min; column oven temperature: 40ºC; mobile phase: methanol:buffer (60:40); 2 ml of formic acid in 1000 ml mobile phase; diluent: methanol:water (50:50) + 2 ml of formic acid in 1000 ml of diluents; retention time: 2.1 min for candesartan (analyte); 1.0 min for propranolol (internal standard); run time: 3.3 min; extraction technique: protein precipitation.

Preparation of standards for calibration and quality control

Accurately transferred about 10 mg of the candesartan working standard into a 10 ml volumetric flask. It was dissolved in 5 ml of methanol and the volume up made up to the mark with methanol to prepare a 1 mg/ml solution. The final concentration of 0.1 mg/ml (100000 ng/ml) was carried out by dilution of 1 ml of the above 1 mg/ml solution up to 10 ml with methanol.

The working solutions of candesartan were prepared using the diluent. The final concentration was made up to 58.270, 116.939, 708.723, 2531.155, 10124.620, 25960.563, 37086.519, 46353.149 and 51509.054 ng/ml. Similarly, the lower quality control (LQC) concentration (162.254 ng/ml), middle quality control (MQC) concentration (16225.352 ng/ml), higher quality control (HQC) concentration (36056.338 ng/ml) and lower limit of quantification (LLOQ; 64.902 ng/ml) samples were prepared. Required numbers of samples of concentration of candesartan ranging from 1 to 1000 ng /ml were prepared by making up the volume with drug-free plasma and labelling them as STD-1 to STD-9, which are 1.169, 2.339, 14.174, 50.623, 202.492, 519.211, 741.730, 927.163 and 1030.181 ng/ml, respectively.

Sample preparation

0.10 ml of sample into was accurately pipetted into prelabeled vials and 500 μl of propranolol (internal standard) was added and mixed for 2 min (for blank sample, 500 μl of methanol solution was added instead of the internal standard solution). Methanol in propranolol solution was used for protein precipitation. Samples were centrifuged at 4800 rpm at less than 10°C for 15 min. Then, 0.4 ml supernatent was transferred into the prelabeled vial containing 0.4 ml diluent and mixed properly. 0.5 μl of this mixture was then injected into an HPLC system using an auto sampler.

The concentration of candesartan and propranolol was calculated from the area ratio v/s spiked plasma concentration regression equations, with reciprocate of the drug concentration as a weighting factor (1/[concentration]^2, i.e. 1/X^2): $y = mx + c$

where, y = peak area ratio of candesartan to Propranolol, m = slope of the calibration curve, x = concentration of candesartan, c = y-axis intercept of the calibration curve

Method validation

The specified LC-MS/MS method was validated to estimate candesartan in human plasma as per the US-FDA guidelines.[11] Various validation parameters, such as linearity, precision, accuracy, specificity,

stability study and matrix effect, were carried out to prove the capability of the proposed method.

Linearity

A calibration curve comprising of a "blank matrix" (matrix processed without analyte and internal standard), a "zero standard" (blank matrix processed only with internal standard) and nine calibration standards covering the expected range were processed and analyzed. The linearity of the developed method was achieved in the range of 1.2–1030 ng/ml ($r^2 = 0.9996$). The present method was capable of quantifying the lower concentration of candesartan accurately [Figure 2]. %nominal values for all the standards were within the limits of 85–115%, except for STD-1, which was between 80 and 120%, as per the US-FDA guidelines.[11]

Accuracy and precision

Calibration standards and six replicates each of LLOQ, LQC, MQC and HQC samples were processed and analyzed as per the procedure described in sample preparation. For intrabatch and interbatch accuracy, %nominal concentration of the back-calculated value for LLOQ, LQC, MQC and HQC, analyzed in a single analytical batch and thee different batches, were calculated respectively as per formula. %nominal concentration was found to be within the criteria of 85–115%.

For intrabatch and interbatch precision, standard deviation and %coefficient of variation for LLOQ, LQC, MQC and HQC samples, analyzed on one batch and five different batches, were calculated, respectively, which were found to be within criteria ≤15, except LLOQ (≤20). Results of the interbatch precision and accuracy study are described in Table 1.

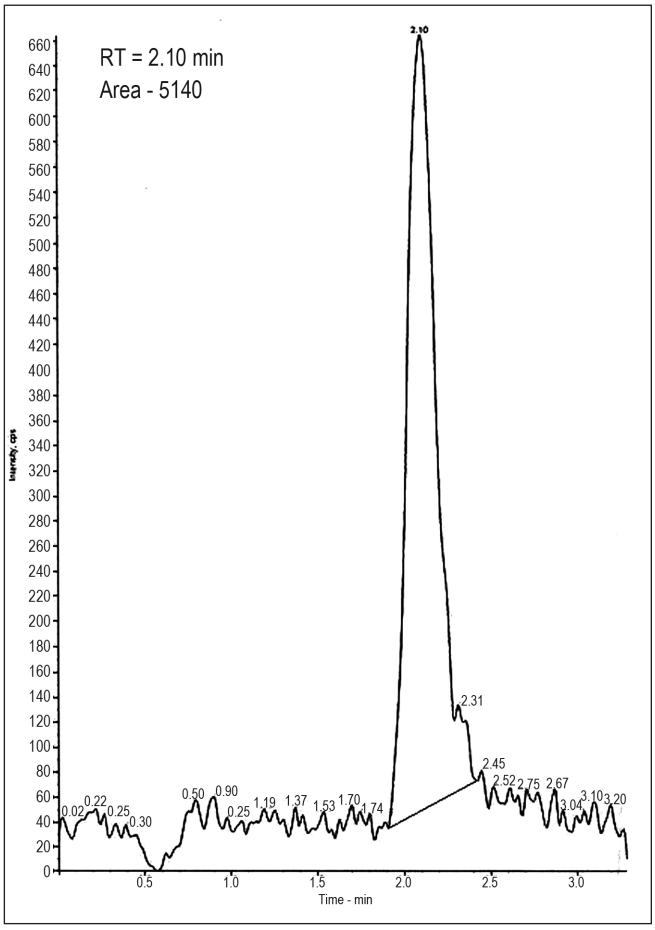

Figure 2: Chromatogram of candesartan in the lower limit of quantification sample

Recovery

Recovery for analyte and internal standard was performed by comparing the area of the extracted samples at three different concentrations (LQC, MQC and HQC) with unextracted standards area that represents 100% recovery. %recovery of an analyte(s) at LQC, MQC and HQC samples and an internal standard were calculated, which were found to be 101.9% for candesartan and 87% for the internal standard (propranolol), as depicted in Table 2.

Specificity and selectivity

Plasma matrix including four normal plasma lots with the anticoagulant, one lipemic plasma and one hemolyzed plasma lot were processed and analyzed. One sample each of the six plasma lots at blank and LLOQ level were processed and analyzed as per the procedure described in sample preparation. Area response at the RT of candesartan in the blank was less than 20% of the LLOQ area response and the area response at the RT of propranolol (internal standard) in the blank plasma was less than 5% of the internal standard area response as per the limit.

Sensitivity

Calibration standards, zero standard (matrix spiked only with internal standard) and six sets of matrix sample spiked at LLOQ concentration using blank matrix lot were processed and analyzed as per the procedure described in sample preparation. Response of candesartan at the LLOQ level was greater than five-times that of the blank plasma. %coefficient of variation (CV) and %nominal concentration were found to be 10.2% and 94.8%, respectively, which passes the limit of %CV (≤20) and %nominal concentration (80–120%).

Table 1: Results of interday and intraday precision

P and A	Interday				Intraday			
	LLOQ (1.298 ng/ml)	LQC (3.245 ng/ml)	MQC (324.51 ng/ml)	HQC (721.13 ng/ml)	LLOQ (1.298 ng/ml)	LQC (3.245 ng/ml)	MQC (324.51 ng/ml)	HQC (721.13 ng/ml)
Mean concentration* ± SD	1.34 ± 11.3	3.437 ± 7.3	324.10 ± 2.7	739.05 ± 2.1	1.44 ± 7.9	3.39 ± 6.7	324.74 ± 3.6	736.96 ± 2.4
Nominal (%)	103.4	100.4	99.9	102.5	111.0	104.5	100.1	102.2

*Average of six determinations, SD = Standard deviation

Table 2: Results of accuracy study

Sample		Candesartan		Propranolol (internal standard) (50.0 ng/ml)	
		Mean area* ± SD	% recovery	Mean area ± SD	%recovery
Extracted	LQC (3.245 ng/ml)	13720 ± 10.9	101.9	354722.2 ± 8.2	87.0
Unextracted		13466.7 ± 6.0		407833.3 ± 1.5	
Extracted	MQC (324.51 ng/ml)	1607500 ± 7.0	102.8		
Unextracted		1563333.3 ± 2.2			
Extracted	HQC (721.13 ng/ml)	3590000 ± 1.6	110.6		
Unextracted		3245000.0 ± 1.4			

*SD = Standard deviation

Table 3: Results of the stability study

Type of stability study	%CV		Mean %change	
Long-term stock solution stability	0.8		0.6	
	LQC (*n* = 6)	HQC (*n* = 6)	LQC (*n* = 6)	HQC (*n* = 6)
Freeze and thaw stability study	3.2	0.9	0.5	2.5
Process stability in auto aampler	6.0	1.1	12.4	4.6
Bench-top stability study	6.4	2.0	1.2	1.1

Dilution integrity

Analyte spiking stock solution was spiked in blank plasma to get a concentration equivalent to three-times of the upper limit of quantification and diluted with blank plasma to get 1/5 and 1/10 concentrations of the spiked sample or as per requirement. Calibration standards and six aliquots each of the diluted samples (1/5 and 1/10 dilutions) were processed and analyzed as per the procedure described in sample preparation. %nominal concentration was found to be 111.97% and 108.3% for both the dilutions, which passed the limit of 85–115%.

Matrix effect

Calibration standards, in the same matrix which was to be used during validation experiment, and three replicates from three different plasma matrices at LQC and HQC levels were processed and analyzed as described in sample preparation. %nominal concentration of LQC and HQC were found to be 100.4% and 106.4%, respectively, which fulfilled the criteria of %nominal concentration (85–115%).

Stability study

Freeze and thaw stability of candesartan

Freeze and thaw stability of the analyte was determined after three freeze and thaw cycles at LQC and HQC levels. Mean %changes were 0.5% and 2.5% for LQC and HQC, respectively. This fulfilled the criteria of mean %change (within 15% as shown in Table 3).

Process stability of candesartan at 6C in an auto sampler for 24 h

Process stability of the analyte is determined at LQC and HQC levels. Mean %changes for LQC and HQC were calculated to be 12.4% and 4.6%, respectively [Table 3].

Bench-top stability of candesartan at room temperature for 6 h

LQC and HQC samples were spiked in human plasma and kept at room temperature for 6 h and were analyzed along with freshly prepared LQC and HQC samples. Mean %changes during the stability period were found to be 1.2% and 1.1% for the LQC and HQC, respectively [Table 3].

Long-term stock solution stability of candesartan at 2–8°C for 6 days

The main stock solution of candesartan was freshly prepared and an aliquot of the stock was kept at 2–8°C for 6 days (stability sample). Aqueous equivalent highest calibration standard of candesartan was

prepared from the stability samples and analyzed. Areas of stability samples and freshly prepared samples were compared to determine the %mean change and %CV. %mean change and %CV were found to be 0.6 and 0.8, respectively [Table 3].

RESULTS AND DISCUSSION

Methanol and ammonium trifluoroacetate buffer were used for preparation of the mobile phase after taking various trials. Buffer concentration was optimized to 1 M after using various concentrations, and formic acid was used to acidify the buffer. The ratio of the buffer was increased to allow for better peak shape and resolution in plasma. Best results were obtained by using the ratio: methanol:buffer (60:40 v/v). The Betasil C8 column was selected to reduce the run time instead of the C_{18} columns. Low flow rate was selected to 0.45 ml/min to increase the efficiency of the column and to reduce the usage of the mobile phase. No interference from endogenous substance was observed in the selectivity exercise at the retention time of candesartan. This is explicit from the chromatogram of the sample [Figure 2] LOQ plasma samples spiked with internal standard. The R-square was consistently 0.99 or greater during the course of the validation. The best fit for calibration curve of chromatographic response versus concentration was determined by the weighted least square regression analysis, with weighting factor of 1/concentration.2 The data of intra- and interday precision and accuracy for candesartan from QC samples are summarized in Tables 1 and 2, respectively. The precision and accuracy of this method conform to the FDA guidance document, which states that the accuracy determined at each concentration level must not exceed 15% (20% for LOQ) and precision must be within 15% (20% for LOQ) of the nominal value. The extraction recoveries from the QC samples at low, middle and high concentrations were 101.9%, 102.8% and 110.6% for candesartan, whereas it was 87% for the internal standard. Recoveries were good, and it was consistent, precise and reproducible with this proposed extraction method [Table 2]. The results from the stability test are presented in Table 3, which demonstrated a good stability of candesartan under the conditions evaluated. Bench-top stability in matrix (7 h), post-preparative (in injector) stability at 5 ± 1°C for 24 h and freeze–thaw stability were determined at six replicates of the low and high QC concentration at -70°C for 24 h, and were found to be acceptable. There was negligible or null matrix factor and matrix effect. Values for all method validation parameters indicate that the methods can be applied for routine bioanalysis.

CONCLUSIONS

The proposed isocratic method is able to estimate a very low concentration of candesartan in human plasma at less-retention time with high recovery compared with the reported method. Therefore, the developed method is simple, rapid, specific, selective, precise and accurate. The protein precipitaion technique used for the extraction purpose made this method time saving.

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