Bioanalytical method development and its validation for determination of candesartan cilexetil by high performance liquid chromatography with UV detection

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Abstract

A simple, sensitive, fast method using isocratic high-performance liquid chromatography (HPLC) and ultra violet detection for the measurement of candesartan in human plasma was developed and validated. The HPLC column, a Phenomenex Luna C8, 250x4.6 mm, 5μ, was equilibrated with an eluent mixture of methanol – 10mM potassium dihydrogen phosphate (pH 3.0) with composition of (85:15 v:v) at 1 mL/min. Each analyte required no longer than 8 minutes. The peaks were eluted at 260 nm wavelength and no interferences found from plasma. Plasma samples were processed using acetonitrile as precipitating agent to extract candesartan. Quantitation was achieved by the measurement of peak area ratio and the absolute recovery varied from 96.92 to 101.07 %. Detection limit for candesartan in plasma was 20 ng/mL. Intra-day coefficients of variation (% CV) ranged from 0.55 to 2.41 and Inter-day (% CV) from 0.94 to 2.45 at three different concentrations.

Keywords: Bioanalytical method, Candesartan cilexetil, Irbesartan, UV-detection.

Introduction

Angiotensin II receptor blockers (ARBs) are a new class of therapeutic agents for hypertension. The ARBs have a more direct mode of action than other drugs affecting the angiotensin converting enzyme inhibitors. Candesartan is a potent, highly selective ARB that is devoid of agonist activity (Belz et al. 1997, Kenakin 1997, Gavras et al. 1999, Zschieke et al. 1999).

Candesartan cilexetil (prodrug) which is a racemic mixture containing one chiral center at the cyclohexyloxy-carbonyloxy- ethyl ester group (Ettinger et al. 2006)

Cilexetil is an abbreviated name of the cyclohexyloxy-carbonyloxy-ethyl moiety that is incorporated into prodrug, candesartan cilexetil to promote the enteric absorption or bioavailability of active parent drug, candesartan. It is hydrolyzed to the active drug immediately on absorption through the intestinal wall by esterase enzyme (Nishikawa et al. 1997, Van et al. 1997, Malerczy et al. 1998, Hideki et al. 2002).

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Therefore hydrolysis of the candesartan cilexetil was carried out in laboratory and separated active moiety candesartan, spiked with drug free human plasma for its determination.

Although various pharmacokinetic studies has been published for candesartan estimation in patients, no detailed chromatographic method by which candesartan was estimated in human plasma, was described (Hubner et al. 1997, Pfister et al. 1999, Gleiter et al. 2002, Teng et al. 2007). We present here a method for candesartan measurement in human plasma based on high-performance liquid chromatography (HPLC) followed by ultraviolet detection. The procedure for the assay involves the treatment of plasma with acetonitrile for total drug determination, and the use of a centrifugal filter device Eltek, Model TC 650 D to separate the unbound drug fraction for free drug determination. The method is reproducible and sensitive, can be used in routine clinical analysis of candesartan cilexetil.

Materials and Method

Candesartan cilexetil is a prodrug and active drug candesartan is available in systemic circulation after hydrolysis in intestinal wall by esterase enzyme. Therefore its hydrolysis was carried out to obtain the active parent drug candesartan. 1 g (1.64 mmoles) of candesartan cilexetil was dissolved in 10 mL acetone. 10 mL of water was added for hydrolysis and concentrated sulphuric acid was added as a catalyst. The reaction mixture was refluxed for 2 hours and 20 minutes. The reaction was monitored by thin layer chromatography (TLC). The mobile phase for TLC was composed of Toluene: Methanol (70:30 v:v). The Rf value for candesartan cilexetil was found to be 0.7 and 0.45 for candesartan. The reaction mixture was poured in crushed ice; solid candesartan was separated, filtered off and recrystallized from methanol. Figure 1 depicts candesartan cilexetil and candesartan.

![Candesartan Cilexetil (Prodrug) and Candesartan (Active drug)](image)

**Figure 1. Candesartan Cilexetil and Candesartan**

Blank plasma (drug free) was obtained from blood bank of KEM hospital, Parel, Mumbai, India. To develop an efficient and reproducible extraction method from human plasma for candesartan, various extraction solvents were attempted (i.e. methanol, methyl acetate, ethyl acetate, diethyl ether, n-Hexane, chloroform and acetonitrile), as the application of liquid-liquid extraction has been shown to be good choice in bioanalysis of drugs using LC-UV assays for other drugs (Jackson 2001).

Various compositions of solvents were tried out but the results were not satisfactory except for acetonitrile (Data not shown). Candesartan showed good solubility in acetonitrile which was further used as precipitating agent. Acetonitrile gave excellent recovery and reproducibility from human plasma for candesartan.
500 μL of drug free plasma was spiked with candesartan and internal standard irbesartan. 1000 μL of acetoafrile as precipitating agent was added to the drug spiked plasma. Final concentration of internal standard irbesartan was 1000 ng/mL. The solutions were vortex mixed for 1 minute and centrifuged at 5000 rpm for 15 min using an Eltek, Model TC 650 D centrifugal device. The upper phase was filtered through 0.45 μm filter and evaporated under nitrogen evaporator. The extracted candesartan was reconstituted in mobile phase (50:50 v:v) and 20 μL of ultra filtrate was then injected into a Phenomenex Luna C8, 250x4.6 mm, 5μ column equilibrated with mobile phase prepared as follows: Methanol-10mM potassium dihydrogen phosphate (pH 3.0) with composition of (85:15 v:v). Total HPLC run time was 7 min; re-equilibration time was 2.5 min. Room temperature 25 °C was maintained throughout the period of analysis. The HPLC system was Perkin Elmer series Binary LC pump, Model 200B/250 equipped with an Ultraviolet detector operating at a wavelength of 260 nm. All operations, such as the injection cycle, were controlled by the Totalchrom program.

**Result and Discussion**

Calibration curve for candesartan (50-5000 ng/mL) were prepared by spiking stock solutions with 500 μL drug free plasma. A linear relationship was obtained between peak area ratio of drug to internal standard irbesartan within entire concentration range; 50-5000 ng/mL. The equation for regression line was: y = 0.0013x + 0.0302, correlation coefficient was > 0.9997 for candesartan. Linearity was confirmed by showing that the slopes of linear calibration curves were statistically different from zero and by comparison of intercept with zero and a correlation coefficient with 1.

Candesartan and irbesartan (internal standard) eluted from the column in 5.94 and 3.72 min, respectively, at a flow rate of 1.0 mL/min. A typical HPLC/UV chromatogram Figure 2a shows a blank plasma chromatogram. Figure 2b shows the chromatogram of irbesartan an internal standard. Figure 2c shows the chromatogram of active drug (candesartan) and irbesartan plasma sample obtained from a spiked sample.

![Figure 2a. Blank plasma](image-url)
Figure 2b. Irbesartan as internal standard in human plasma.

Figure 2c. Chromatogram of candesartan and irbesartan (I.S.) in human plasma.
We assessed the accuracy and precision of the method by intra-day and inter-day validation. Repetability was studied by performing six replicate analyses at high, middle and low concentrations against a calibration curve (50-5000 ng/mL). Table 1a reports accuracy and precision of candesartan measurement by assaying on 5 different days. The accuracy and precision were expressed in % bias and coefficient variation respectively.

<table>
<thead>
<tr>
<th>Nominal concentration (ng/mL)</th>
<th>Measured concentration (ng/mL)</th>
<th>Accuracy(^a) (%Bias)</th>
<th>Precision (%)</th>
<th>Measured concentration (ng/mL)</th>
<th>Accuracy(^a) (%Bias)</th>
<th>Precision (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>48.66</td>
<td>-2.68</td>
<td>0.55</td>
<td>48.17</td>
<td>-3.67</td>
<td>0.94</td>
</tr>
<tr>
<td>500</td>
<td>476.97</td>
<td>-4.60</td>
<td>2.41</td>
<td>531.81</td>
<td>6.36</td>
<td>6.41</td>
</tr>
<tr>
<td>5000</td>
<td>5145</td>
<td>2.9</td>
<td>1.19</td>
<td>5051</td>
<td>1.03</td>
<td>2.45</td>
</tr>
</tbody>
</table>

\(^a\) measured concentration-nominal concentration) x 100/nominal concentration

The extraction efficiency (recovery) was determined assaying drug-free plasma spiked with known amounts of drugs (50, 500 and 5000 ng/mL for candesartan). Each sample was determined six times. In Table 1b the mean analytical recoveries for candesartan are shown. The accuracy of the method was expressed as percent deviation of observed concentration from theoretical concentration with the relative error.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Candesartan concentration (ng/mL)</th>
<th>Average recovery (%)(n=6)</th>
<th>RSD of recoveries (n=6)</th>
<th>Relative error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Plasma</td>
<td>50</td>
<td>97.88±1.22</td>
<td>1.19</td>
<td>2.12</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>96.92±3.65</td>
<td>3.77</td>
<td>3.08</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>101.07±1.27</td>
<td>1.25</td>
<td>-1.07</td>
</tr>
</tbody>
</table>

The lower limit of detection (LLOD) and lower limit of quantitation (LLOQ) for candesartan plasma samples, defined as the concentration resulting in a peak area of three times and ten times the signal-to-noise ratio respectively. The LLOD and LLOQ were 20 ng/mL and 50 ng/mL respectively.

Drug stability in biological fluids is a function of the sample storage conditions, the chemical properties of the drug, the plasma and the container system. The stability of candesartan was evaluated under the conditions reflecting situations likely to be encountered during actual sample collection, handling and analysis. Candesartan was stable for short (24 h) and long terms (90 days) storage, after three freeze-thaw cycles and during the analytical process. Stability studies were carried out as per bio-analytical method validation, ICH guideline (FDA 2001). Table 1c illustrated that all the results from the stability study was under the acceptance range of ± 15%.
Table 1c. Stability of candesartan in human plasma.

<table>
<thead>
<tr>
<th>Test of stability</th>
<th>Candesartan (Accuracy)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HLOQ (5000 ng/mL)</td>
</tr>
<tr>
<td>Freeze-thaw stability</td>
<td>99.98±1.74</td>
</tr>
<tr>
<td>Long term stability</td>
<td>98.86±0.67</td>
</tr>
<tr>
<td>Short term study</td>
<td>97.90±3.19</td>
</tr>
<tr>
<td>Post preparative study</td>
<td>99.66±0.53</td>
</tr>
</tbody>
</table>

In patients, administration of 32mg candesartan cilexetil suspension and tablet showed maximum plasma concentration (Cmax) 282.92 and 238.92 ng/mL respectively (Pfister et al.1999). The developed method was shown the limit of quantitation 50ng/mL which is less than the Cmax in reported pharmacokinetic study of candesartan. Therefore the developed bioanalytical method is suitable for pharmacokinetic study of candesartan cilexetil.

Conclusion

This HPLC assay method for candesartan cilexetil is simple, fast, precise, sensitive and accurate with UV detection for quantification of candesartan in human plasma. In addition, the method requires low sample volumes, providing a reliable determination of candesartan in human plasma, thus allowing the accurate determination of candesartan in hypertensive patients receiving candesartan cilexetil therapy or the method is applicable for estimation of several pharmacokinetic parameters in therapeutic drug monitoring.

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References


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