A dissolution test can identify formulation and manufacturing factors of production batches, in order to ensure in vitro similarity in relation to clinical batch approved with in vivo study (1). Assay is the content or potency of drug substance, defining its existing amount inside dosage form (2). Uniformity of dosage units is the degree of uniformity of drug substance amount among dosage units. Content uniformity test is applied to uncoated tablets, containing ratio of drug substance less than 25 mg or 25% (3).

Candesartan cilexetil (Fig. 1), ((±)-1-(cyclohexyloxycarbonyloxy)ethyl-2-ethoxy-1-[[2’-(1H-tetrazol-5-yl)biphenyl-4-yl][methyl]-1H-benzimidazole-7-carboxylate), is used for treatments of hypertension (high blood pressure) and heart failure (4). It is practically insoluble in water, sparingly soluble in methanol and highly hydrophobic (5–8). Dissolution of immediate release candesartan cilexetil 32 mg tablets is performed in 0.05 M phosphate buffer, pH 6.5 including 0.70% (w/v) polysorbate 20. Validation studies met acceptance criteria of system suitability, specificity, linearity and range, accuracy, precision, detection limit (LOD), quantitation limit (LOQ) and robustness parameters.

Keywords: assay, candesartan cilexetil, content uniformity, dissolution, method validation, tablet

Figure 1. Candesartan cilexetil (CAS number 145040-37-5)
For this reason, HPLC analytical method testing assay, content uniformity and dissolution of candesartan cilexetil tablets in 32 mg dose were aimed for development and validation in this study. Method validation studies were done according to system suitability, specificity, linearity and range, accuracy, precision, LOD, LOQ and robustness parameters (12-20).

EXPERIMENTAL

Chemicals and reagents

The reagents provided from the following suppliers were all of analytical grade: Polysorbate 20 (Tween 20, Merck-Schuchardt, Germany), trifluoroacetic acid (Uvasol, Merck-Schuchardt, Germany), methanol, acetonitrile (Merck, Germany), sodium dihydrogen phosphate dihydrate (NaH2PO4.2H2O, Emprove, Merck, Germany), potassium dihydrogen phosphate (KH2PO4) and sodium hydroxide (NaOH, Riedel-de Haën, Germany) and hydrochloric acid (HCl, J.T. Baker, Holland). Cronus 0.45 µm nylon 25 mm syringe filters were purchased from SMI LabHut Ltd. (UK). Advantec 0.45 µm cellulose acetate 25 mm syringe filters were purchased from Advantec MFS, Inc. (USA). Membrane 0.45 µm high volume (HV) filters were purchased from Merck Millipore Corp. (Germany). All aqueous solutions were prepared with HPLC grade type I water, obtained in-house, from a Milli-Q Gradient A-10 water purification system (Merck Millipore Corp., Germany).

The tablet ingredients were all pharmaceutical grade: Candesartan cilexetil (lot no: 5251-10-017, XunQiao, LinHai, Zhejiang, China), lactose monohydrate (Pharmatose 90M, DMV - Fonterra Excipients, Germany), polyethylene glycol 4000 (Merck-Schuchardt, Germany), hydroxypropyl cellulose (Klucel LF – Pharm, Ashland, USA), starch maize (Roquette, France), carboxymethyl cellulose calcium (Nichirin Chemical Ind., Japan) and magnesium stearate (Peter Greven, Germany).

Certified candesartan cilexetil drug substance was used as working standard. Immediate release candesartan cilexetil 32 mg tablets were prepared with above tablet ingredients. Placebo tablets were also prepared for validation studies, excluding candesartan cilexetil.

Instrumentation and chromatographic conditions

The chromatographic system used to develop assay, content uniformity and dissolution methods was Agilent Technologies HP 1200 series having UV detector and auto sampler (USA). The chromatographic conditions differed only in mobile phases, whereas the others remained constant.

Chromatographic separations were achieved on cyano column (250 × 4.6 mm, 5 µm particle; Cliepeus, Higgins Analytical Inc., USA). The mobile phases were pumped through the column at a flow rate of 1.0 mL/min, maintaining temperature at 25°C. The injection volume to carry out the chromatography was set at 20 µL. The UV detection wavelength (λ) was set at 254 nm. Assay and content uniformity test required 13 min and dissolution test required 12 min for separation.

The mobile phase for assay and content uniformity test consisted of 40% 0.05 M phosphate buffer solution, pH 4.5 and 60% methanol adjusted to pH 4.0 with trifluoroacetic acid. To prepare 0.05 M phosphate buffer solution, pH 4.5, 6.8 g KH2PO4 was weighed, diluted to 1000 mL with HPLC grade water and dissolved for 5 min by mixing.

The mobile phase for dissolution test consisted of 50% acetonitrile and 50% 1 mM phosphate buffer solution adjusted to pH 2.0 with trifluoroacetic acid. To prepare 1 mM phosphate buffer solution, 136.08 mg KH2PO4 was weighed, diluted to 1000 mL with HPLC grade water and dissolved for 5 min by mixing.

The buffer solutions and mobile phases were filtered through HV-membrane filter.

The assay of tablet samples was calculated using equation 1:

\[ \text{Drug amount (mg)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times \frac{\text{concentration (standard)}}{\text{volume (sample)}} \times \frac{\text{mean tablet weight}}{\text{weight (sample)}} \times \text{mean tablet weight} \]  

(Eq. 1)

where, A (sample) is the measured peak area of sample solution, A (standard) is the measured peak area of standard solution of candesartan cilexetil, concentration (standard) is the concentration of standard solution of candesartan cilexetil (mg/mL), volume of sample solution is 50 mL, mean tablet weight is 260 mg, weight of sample is equivalent to mean tablet weight (mg), taken from mixture of 5 powdered tablets.

The content of tablet samples was calculated using equation 2:

\[ \text{Drug content (%) } = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times \frac{\text{concentration (standard)}}{\text{volume (sample)}} \times \frac{\text{mean tablet weight (mg)}}{L} \times 100 \]  

(Eq. 2)

where, L is drug load of 32 mg and weight of sample is the tablet weight (mg).

A calibrated dissolution apparatus (Sotax AG, Switzerland) was used with paddles at 50 rotations per minute (rpm) and bath temperature maintained at 37 ± 0.5°C. Freshly prepared 0.05 M phosphate
buffer, pH 6.5 (900 mL) including 0.70% (w/v) polysorbate 20 was used as dissolution medium. To prepare the dissolution medium, 7.8 g NaH₂PO₄ × 2H₂O was weighed, dissolved in about 900 mL of HPLC grade water and pH was adjusted to 6.5 ± 0.05 with 400 g/L NaOH reference solution. Finally, it was diluted up to 1000 mL with additional HPLC grade water. Seven grams polysorbate 20 was added to prepared buffer solution and mixed for 10 min. In each dissolution test, 6 tablets were evaluated. Tablet sample solutions were collected at 10, 20, 30, 45 and 60 min (9). At each time point, 5 mL sample was removed from each vessel using a glass pipette and equal volume of fresh medium was added to each vessel to maintain the volume constant. After filtration through nylon filter, each sample was transferred to vial and analyzed.

The dissolved amount of candesartan cilexetil from tablet samples was calculated using equation 3:

\[ \text{Dissolved} \% = \frac{900L \times A \text{ (sample)}}{A \text{ (standard)}} \times \text{concentration (standard)} \times 100 \quad (\text{Eq. 3}) \]

### Preparation of working standard solutions

Standard solution of candesartan cilexetil for assay and content uniformity test was prepared at 0.64 mg/mL amount corresponding to 100% concentration. Candesartan cilexetil (32 mg) was weighed, placed in a 50 mL volumetric flask and diluted up to 50 mL with methanol. This solution was sonicated for 10 min and passed through cellulose acetate filter.

Standard solution of candesartan cilexetil for dissolution test was prepared at 35.56 µg/mL amount corresponding to 100% concentration. Candesartan cilexetil (35.56 mg) was weighed, placed in a 50 mL volumetric flask and diluted up to 50 mL with methanol. This solution was sonicated for 10 min and passed through cellulose acetate filter.

### Preparation of calibration standard solutions

The range in which linearity is evaluated for assay test is 80-120%, for content uniformity is 60-140%, for dissolution test is 60-120% of the test concentration, including at least 5 levels (12, 17).

Sample solutions prepared for assay and content uniformity test were in levels of 60% (0.384 mg/mL), 80% (0.512 mg/mL), 100% (0.64 mg/mL), 120% (0.768 mg/mL), 140% (0.896 mg/mL), 160% (1.024 mg/mL) and 180% (1.152 mg/mL) of the working standard solution.

The related amount of candesartan cilexetil and 228 mg placebo were weighed, placed in a 50 mL volumetric flask, diluted up to 50 mL with methanol and filtered. Each placebo solution was transferred to vial and analyzed in duplicate.

### Preparation of placebo solutions

For assay and content uniformity test 10 placebo tablets were crushed with mortar hand, 228 mg powdered placebo (mean tablet weight-drug load) was weighed, placed in a 50 mL volumetric flask and diluted up to 50 mL with methanol. It was sonicated for 10 min. In order to prepare 10% (v/v) placebo solution in methanol, 10 mL of the solution was transferred using a glass pipette into 100 mL volumetric flask, diluted up to 100 mL with methanol and passed through cellulose acetate filter.

For dissolution test 10 placebo tablets were crushed with mortar hand, 228 mg powdered placebo was weighed, diluted to 900 mL with dissolution medium in a volumetric flask. It was held for 60 min in shaking water bath conditioned to 37 ± 0.5°C and then passed through nylon filter.

Each placebo solution was transferred to vial and analyzed in duplicate.

### Preparation of sample solutions

In validation study, accuracy is evaluated using at least 3 levels/3 replicates covering the specified range (12, 17).

Sample solutions prepared for assay and content uniformity test were in levels of 60% (0.384 mg/mL), 80% (0.512 mg/mL), 100% (0.64 mg/mL), 120% (0.768 mg/mL), 140% (0.896 mg/mL), 160% (1.024 mg/mL) and 180% (1.152 mg/mL) of the working standard solution.

Related amount of candesartan cilexetil and 228 mg placebo were weighed, placed in a 50 mL volumetric flask, diluted up to 50 mL with methanol, sonicated for 10 min and passed through cellulose acetate filter.

Sample solutions prepared for dissolution test were in levels of 60% (21.336 µg/mL), 70% (24.892 µg/mL), 80% (28.448 µg/mL), 90% (32.004 µg/mL), 100% (35.56 µg/mL), 110% (39.116 µg/mL), 120% (42.672 µg/mL) and 130% (46.232 µg/mL) of the working standard solution.
up to 50 mL with methanol and sonicated for 10 min. Five mL was transferred using a glass pipette into 100 mL volumetric flask, diluted up to 100 mL with 0.05 M phosphate buffer, pH 6.5 including 0.70% (w/v) polysorbate 20 and passed through nylon filter.

Each sample solution was transferred to vial and analyzed in duplicate.

Method validation studies
System suitability
System suitability standard solution was working standard solution, prepared in 0.64 mg/mL for assay and content uniformity test and 35.56 µg/mL for dissolution test corresponding to 100% concentration. System suitability was determined from 5 replicate injections of the system suitability standard before sample analyses. The acceptance criteria were; number of theoretical plates (N) > 2000, tailing factor (symmetry factor, Aₜ) of 0.8 < Aₜ < 1.5, relative standard deviation (RSD) ≤ 1.5% for peak area of assay and content uniformity test and RSD ≤ 2.0% for peak area of dissolution test (13, 16).

Specificity
For the specificity study of assay and content uniformity test, identification of candesartan cilexetil was studied comparing the chromatograms of system suitability standard solution, 10% (v/v) placebo solution in methanol, methanol as solvent and 10% (v/v) tablet sample solution in methanol. In order to prepare 10% (v/v) tablet sample solution in methanol, 10 tablets were crushed with mortar and hand and 260 mg was weighed, placed in a 50 mL volumetric flask, diluted up to 50 mL with methanol and sonicated for 10 min. Ten mL of the solution was transferred using a glass pipette into 100 mL volumetric flask, diluted up to 100 mL with methanol and passed through cellulose acetate filter.

For the specificity study of dissolution test, identification of the candesartan cilexetil was studied comparing the chromatograms of system suitability standard solution, placebo solution, dissolution medium and tablet sample solution taken from dissolution study.

All chromatograms were examined to check the absence of interference between candesartan cilexetil, both with placebo solution (excipients) and methanol or dissolution medium (12).

Linearity and range
To obtain the calibration line for assay test, 5 levels of calibration standard solutions over 80-120% (0.512 mg/mL, 0.576 mg/mL, 0.64 mg/mL, 0.704 mg/mL, 0.768 mg/mL) were prepared.

To obtain the calibration line for content uniformity test, 7 levels of calibration standard solutions over 60-140% (0.384 mg/mL, 0.512 mg/mL, 0.576 mg/mL, 0.64 mg/mL, 0.704 mg/mL, 0.768 mg/mL, 0.896 mg/mL) were prepared.

To obtain the calibration line for dissolution test, 7 levels of calibration standard solutions over 60-120% (21.336 µg/mL, 24.892 µg/mL, 28.448 µg/mL, 32.004 µg/mL, 35.56 µg/mL, 39.116 µg/mL, 42.672 µg/mL) were prepared.

The analytical ranges were established for obtaining acceptable linearity, accuracy and precision. The data of peak area versus drug concentration were treated by linear least square regression analysis. The experimental results were represented graphically, obtaining a calibration graph and a calibration equation. The acceptance criterion was regression coefficient (r) ≥ 0.999 (16).

Accuracy
The accuracy of assay test was operated as described in sample solution preparation at levels of 80% (3 samples), 100% (3 samples) and 120% (3 samples), where a known amount of the drug was added to a determined amount of placebo and 9 individual samples were prepared. For accuracy testing of content uniformity, 60% (3 samples) and 140% (3 samples) levels were included and 6 individual samples were additionally prepared.

The accuracy of dissolution test was operated as described in sample solution preparation at levels of 60% (3 samples), 90% (3 samples) and 120% (3 samples) and 9 individual samples were prepared. Recovered candesartan cilexetil amount was calculated in relation to the added amount. The acceptance criteria for each recovery was 98-102% and for RSD of each level (3 samples) was ≤ 2.0% for assay and content uniformity test, whereas for dissolution test the acceptance criteria for each recovery was 95-105% and for RSD of each level (3 samples) was ≤ 3.0% (12, 19).

Precision
In the precision study, repeatability and intermediate precision tests were carried out.

For repeatability testing of assay and content uniformity, 6 consecutive tablet sample solutions were prepared. In order to prepare tablet sample solution in methanol, 10 tablets were crushed with mortar and hand and 260 mg was weighed, placed in a 50 mL volumetric flask, diluted up to 50 mL with methanol and sonicated for 10 min. Tablet sample solution was passed through cellulose acetate filter, transferred to vial and analyzed. For repeatability
Figure 2. Chromatogram of system suitability (a); Chromatogram of 10% (v/v) placebo solution in methanol (b); Chromatogram of methanol (c); Chromatogram of 10% (v/v) tablet sample solution in methanol (d); for assay and content uniformity test
testing of dissolution, 6 consecutive tablet sample solutions were taken from the same vessel at 30 min. Each tablet sample solution was passed through nylon filter, transferred to vial and analyzed. The acceptance criterion for RSD of 6 tablet sample solutions for repeatability testing was $\leq 2.0\%$.

For testing the intermediate precision, study of the variability took place with the same analyst, the same column and the same equipment on 2 different working days. The procedure of assay and content uniformity test was realized following up the repeatability testing. For testing the intermediate

![Figure 3. Chromatogram of system suitability standard (a); Chromatogram of placebo solution (b); Chromatogram of dissolution medium (c); Chromatogram of tablet sample solution taken from dissolution study (d); for dissolution test](image)
precision of dissolution test, 6 different tablet sample solutions were taken from 6 different vessels at 30 min. The difference between mean recovery % of 2 days (Δ) for intermediate precision testing was calculated (12).

**LOD and LOQ**

LOD and LOQ of the tests were calculated using equations 4 and 5:

\[
\text{LOD} = 3.3 \frac{\sigma}{S} \quad \text{(Eq. 4)}
\]

\[
\text{LOQ} = 10 \frac{\sigma}{S} \quad \text{(Eq. 5)}
\]

where, \( \sigma \) is standard deviation (SD) of the response and S is the slope of the calibration curve (12).

**Robustness**

The study of robustness was carried out to evaluate the influence of variations in the chromatographic conditions and the stability of standard and sample solutions.

The factors chosen for chromatographic separation and stability study of sample solution preparation were temperature (°C + 10°C), pH (± 0.1), salt concentration (KH₂PO₄ % ± 10%), organic compound concentration (methanol % ± 3% or acetonitrile % ± 2.5%) and flow rate (mL/min ± 0.1) of mobile phase (13, 18). The acceptance criteria of system suitability parameters for chromatographic separation were applied.

The stability of standard and sample solutions was evaluated at 0, 24, 48 h for assay and content uniformity test and at 0, 12, 24 h for dissolution test held in amber glass at ambient temperature. The acceptance criterion for recovery was 98-102% for assay and content uniformity test and 97-103% for dissolution test, calculated using equation 6:

\[
\text{Recovery of sample solution (％) } = \frac{C_s \times 100}{C_0} \quad \text{(Eq. 6)}
\]

where, \( C_0 \) is the concentration of analyte at the beginning and \( C_s \) is the concentration of analyte at sampling time (12, 19).

**RESULTS**

**System suitability**

Candesartan cilexetil peaks appear at 10.1 min for assay and content uniformity test and at 10.4 min for dissolution test (Figs. 2, 3). Mean peak area of 20 421, A, of 1.339 and N of 12 238 with their RSD of 0.041%, 0.202% and 0%, respectively, were determined for assay and content uniformity test, mean peak area of 1 115.1, A, of 0.851 and N of 57 865 with their RSD of 0.037%, 0.134% and 0.878%, respectively, were determined for dissolution test.

**Specificity**

No major peak other than candesartan cilexetil in both methanol and dissolution medium was observed, there was no interference between excipients. Candesartan cilexetil was noticed to be eluted from the ingredients (Figs. 2, 3).

**Linearity and range**

The calibration lines for assay, content uniformity and dissolution tests are shown in Figure 4. The equation of calibration line for assay test was found to be \( y = 32.277x - 14.682 \) with \( r \) of 0.9997; \( r^2 \) of 0.9994 was obtained for the standard curve within 95% confidence interval (CI). The equation of calibration line for content uniformity test was found to be \( y = 32.831x - 152.15 \) with \( r \) of 0.9994; \( r^2 \) of 0.9988 was obtained for the standard curve within

<table>
<thead>
<tr>
<th>Concentration level</th>
<th>Assay and content uniformity test</th>
<th>Dissolution test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovery (%)</td>
<td>Recovery (%)</td>
</tr>
<tr>
<td>Sample</td>
<td>60%</td>
<td>80%</td>
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<tr>
<td>1</td>
<td>99.37</td>
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<tr>
<td>Mean (%)</td>
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<tr>
<td>SD*</td>
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<td>1.108</td>
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<tr>
<td>RSD** (%)</td>
<td>1.330</td>
<td>1.114</td>
</tr>
<tr>
<td>CI*** (α**** = 0.05)</td>
<td>± 3.332</td>
<td>± 2.753</td>
</tr>
</tbody>
</table>

*SD: Standard deviation. **RSD: Relative standard deviation. ***CI: Confidence interval. ****α: Level of significance.
The equation of calibration line for dissolution test was found to be \( y = 31.828x - 5.2 \), with \( r \) of 0.9992; \( r^2 \) of 0.9985 was obtained for the standard curve within 95% CI.

**Accuracy**
The recovery results obtained for accuracy of assay, content uniformity and dissolution tests are presented in Table 1. The recovery results for assay test ranged from 98.41 to 100.76% and RSD ranged from 0.359 to 1.228%. The recovery results for content uniformity test ranged from 99.06 to 101.99% and RSD ranged from 0.929 to 1.330%. The recovery results for dissolution test ranged from 99.18 to 100.60% and RSD ranged from 0.035 to 0.594%.

**Precision**
The precision study results are presented in Table 2. Assay and content uniformity test had mean recovery of 100.76% and RSD of 0.694% for repeatability testing corresponding to the first day, mean recovery of 102.27% and RSD of 0.312% for intermediate precision corresponding to the second day.

Dissolution test had mean recovery of 94.10% and RSD of 1.844% for repeatability testing corresponding to the first day, mean recovery of 98.04% and RSD of 7.418% for intermediate precision corresponding to the second day.

The RSDs obtained for repeatability testings were below 2.0%. The difference between mean recovery results of individual days was obtained to be 1.51% (\( \Delta = 102.27 - 100.76\% \)) for assay and content uniformity test, whereas for dissolution test it was obtained to be 3.94% (\( \Delta = 98.04 - 94.10\% \)). RSD of 7.418% for intermediate precision was obtained to be below 10.0% (20).

**LOD and LOQ**
LOD result obtained for assay test was 0.009 mg/mL, for content uniformity test was 0.022 mg/mL and for dissolution test was 1.060 µg/mL.

LOQ result obtained for assay test was 0.028 mg/mL, for content uniformity test was 0.066 mg/mL and for dissolution test was 3.211 µg/mL.

**Robustness**
Small variations of chromatographic conditions were obtained, but all of them met the acceptance criteria of system suitability parameters.

The recovery results at 48 h of working standard and tablet sample solutions for assay and content uniformity test were obtained to be 99.81 and 101.03%, respectively, within acceptable limits.

For dissolution test, the recovery result at 12 h of working standard solution was obtained to be 97.60% within acceptable limits; where the recovery result at 12 h of tablet sample solution was 96.31%, out of limits.

**DISCUSSION AND CONCLUSION**
The development of analytical methods started with selecting the chromatographic conditions for...
Development and validation of HPLC analytical methods used for... 365
candesartan cilexetil determination in tablets. Dissolution method was firstly developed, by mainly modifying of mobile phase KH₂PO₄ salt and acetonitrile ratios for sufficient system suitability parameters. The optimum conditions were reached after evaluation of minimum retention time and good chromatogram separation of candesartan cilexetil. Chromatographic conditions of dissolution method were determined to be: mobile phase organic compound concentration 50% acetonitrile, mobile phase salt concentration 50% 1 mM phosphate buffer solution, mobile phase pH 2.0, flow rate 1.0 mL/min,

![Graphs](image)

Figure 4. Calibration line for assay test (a); Calibration line for content uniformity test (b); Calibration line for dissolution test (c)
temperature 25°C, injection volume 20 µL, UV detection wavelength 254 nm. Developed assay and content uniformity method differed in mobile phase composition, whereas the other parameters remained the same. In respect of solubility, methanol was chosen as organic solvent instead of dissolution medium and as mobile phase organic compound instead of acetonitrile. The necessity of changing the mobile phase composition was the lack of chromatogram separation, when dissolution medium was removed; for which a new solvent such as methanol in which candesartan cilexetil is soluble was needed. The mobile phase composition for assay and content uniformity method was specified after obtaining sufficient system suitability parameters; thus, mobile phase organic compound concentration 60% methanol, mobile phase salt concentration 40% 0.05 M phosphate buffer solution, pH 4.5 and mobile phase pH 4.0 were set as optimum conditions.

This work has proposed a common assay and content uniformity method and a dissolution method used for evaluating immediate release candesartan cilexetil 32 mg tablets, complying with validation requirements. It has been proved that the common assay and content uniformity method was specific, linear between 60 and 140% of the working concentration (0.64 mg/mL) for candesartan cilexetil with r square higher than 0.999, exact, precise, accurate and robust regarding the stability of solutions and applied chromatographic conditions across the analytical range. The dissolution method was also concluded to be: specific, linear between 60 and 120% of the working concentration (35.56 µg/mL) for candesartan cilexetil with r square higher than 0.999, exact, precise, accurate and robust regarding the applied chromatographic conditions across the analytical range. Tablet sample solution taken from dissolution study was concluded to be analyzed without waiting.

Thus, the validated methods were successfully established for quality control purposes of developed tablets.

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