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tion requires large quantities of toxic solvents and additional evaporation of the extract, which makes this method too time-consuming for the preparation of samples. With some of the methods\textsuperscript{6,8,9} a long retention time of candesartan is obtained, resulting in long analysis, which makes the studies with a large number of samples very time-consuming.

The here presented method was developed for the determination of candesartan in human plasma. To be adequate for the intended purpose, the method has to be sensitive, accurate and precise, which was all confirmed by its validation according to the requirements proposed by Viswanathan et al.\textsuperscript{10} and OECD Principles of Good Laboratory Practice.\textsuperscript{11} Candesartan from human plasma was extracted by solid-phase extraction which is a very efficient technique for bioanalytical sample preparation. LC-tandem mass spectrometry with electrospray ionization in positive mode and selected reaction monitoring (SRM) mode was used for the analysis.

This article proposes the use of isotopically labeled internal standard candesartan-d\textsubscript{4}, which has been proven to provide results with excellent precision and accuracy and which can be used with advantage for a reliable and accurate measurement of candesartan in human plasma.

2. Experimental

2.1. Chemicals and Materials

The reference standard of candesartan (98.5\%) was obtained from Krka, d.d., Novo mesto (Slovenia). Candesartan-d\textsubscript{4} (97.3\%), which was used as the internal standard, was purchased from Synfine Research (Canada).

Methanol and water were obtained from Merck (Germany), acetonitrile and formic acid from J. T. Baker (The Netherlands) and ammonia solution and sodium hydroxide from Fluka (Germany). All organic solvents were of HPLC grade and chemicals and reagents were of analytical grade. Heparinized drug-free human plasma (including a batch of hemolytic and lipemic plasma) was supplied by Trina Bioreactives AG (Switzerland) and stored at –20 °C until used.

The test formulation was the 32 mg Candesartan tablet.

2.2. Instrumentation

RapidTrace SPE Workstation (Zymark, USA) was used for automated solid-phase extraction procedures (SPE).

Liquid chromatograph (LC) Agilent Technologies 1200 Series with an autosampler PAL HTC CTC Analytics hyphenated to tandem triple quadrupole mass spectrometer (MS/MS) MDS-Sciex API 4000 was used for the LC-MS/MS analysis. Peak area ratios of candesartan versus the internal standard were processed by Analyst software version 1.4.2.

2.3. LC-MS/MS Conditions

Chromatographic separation was obtained on a Gemini C18 110Å column (50 mm x 2 mm internal diameter (ID), 5 μm; Phenomenex (USA) preceded by a guard column Gemini C18 110Å (4 mm x 2 mm ID, 5 μm; Phenomenex) operating at +20 °C. The autosampler temperature was kept at +5 °C. The mobile phase consisted of acetonitrile and 5 mM ammonium formate (90:10, v/v). The flow rate was 0.3 mL/min. The total run time was 2 min and the retention time was 0.58 min for candesartan and internal standard. The injection volume was 10 μL.

Mass spectrometer was operated in the positive ionization mode. Optimization of MS/MS method was performed with the “Infusion Optimization” where a direct injection of 10 ng/mL solution of candesartan and internal standard was carried out while monitoring the characteristic mass transitions. The compound-specific parameters such as collision energy, declustering potential and collision exit potential were optimized. The SRM mode was used for detection and quantitation. The SRM transitions at m/z 441.3 > 263.1 for candesartan and m/z 445.5 > 267.1 for internal standard were used for the quantification of candesartan and internal standard, respectively. The mass spectra are presented in Figure 1. Quantitation was performed using weighted linear regression analysis (1/conc.) of peak area ratios of candesartan and internal standard.

2.4. Standard Solutions, Calibration Curve (CC) and Quality Control (QC) Samples

Candesartan and internal standard (candesartan-d\textsubscript{4}) stock standard solutions at concentrations of 1 mg/mL and 100 μg/mL, respectively, were prepared in methanol. Working standard solutions for candesartan and internal standard were prepared from stock standard solution using methanol solution as diluent. All standard working solutions were kept at +5 °C. From them, we prepared samples for the calibration curve (CC) within the range of 1 to 400 ng/mL (1, 2, 5, 10, 25, 50, 100, 200, 300, 400 ng/mL) and for quality control (QC) samples at four candesartan concentration levels (3, 15, 75, 280 ng/mL).

2.5. Sample Preparation

Strata X extraction cartridges, 60 mg/3 mL, were obtained from Phenomenex (USA). A 500 μL aliquot of human blank plasma sample was mixed with 100 μL of internal standard solution (500 ng/mL), 100 μL of candesartan working standard solutions for calibration curve or quality control samples and 500 μL of water. In sample
preparation for study samples, the same procedure was followed, except for the addition of candesartan working standard, which was replaced by addition of 100 μL of methanol. The prepared sample was loaded onto a Strata X extraction cartridge that had been first pre-conditioned with 2.0 mL of acetonitrile and then twice with 2.0 mL of methanol.

Figure 1: Product mass spectra (MS/MS) and chemical structure of (A) candesartan and (B) candesartan-d4.
water. After the washing with 2.0 mL of water, candesartan and internal standard were eluted with 1.5 mL of acetonitrile. All plasma samples, including the blank samples, samples for the calibration curve, quality control samples and the samples for validation were treated using the same sample preparation procedure. The extracts (10 μL) were injected into the LC-MS/MS system.

3. Results and Discussion

The presented method was validated for specificity, lower limit of quantification, linearity, precision and accuracy, matrix effect, matrix factor, sample dilution, sample preparation recovery, hemolysis effect, solution stability, stability in plasma and carry-over test performance. All validation procedures were performed according to the acceptance criteria for bioanalytical method validation. All acceptance criteria are presented in Table 1.

3.1. Specificity

Specificity was demonstrated by using six independent sources of blank matrix, which were individually analyzed and evaluated for the presence of any interference. When the chromatograms of the plasma samples spiked with candesartan and internal standard (see supplementary material S1–S6) were compared with the chromatograms of the blank plasma samples, no significant interference was observed in the six blank matrix samples at the retention times and mass transitions of candesartan or internal standard.

Table 1: Acceptance criteria for validation parameters.

<table>
<thead>
<tr>
<th>Validation parameter</th>
<th>Acceptance criteria according to Viswanathan et al.\textsuperscript{10} and internal requirements of the bioanalytical laboratory:</th>
</tr>
</thead>
</table>
| Specificity          | N≥6  
 at least 2/3 samples <30.0\% of peak area at LLOQ and ≤1/3 samples 30.0–50.0\% of peak area at LLOQ  
 all samples <5.0\% of mean peak area of internal standard at CC |
| Sensitivity (LLOQ)   | N≥6  
 CV≤20.0\%  
 Accuracy: 80.0–120.0\% |
| Linearity (CC samples) | N≥6, R\(^2\) >0.9800  
 CV≤15.0\% (20.0\% at LLOQ)  
 Accuracy: 85.0–115.0\% (80.0–120.0\% at LLOQ) |
| Precision and accuracy | N≥6  
 inter-day and intra-day  
 CV≤15.0\%  
 Accuracy: 85.0–115.0\% |
| Matrix effect        | N≥6  
 CV≤15.0\%  
 Accuracy and Accuracy (mean): 85.0–115.0\%  
 Internal standard response must be within ± 80.0\% of mean internal standard area  
 At least in 75\% of all matrices, the accuracy of 67\% of all QC samples should be between 85.0–115.0\%  
 Accuracy of 75\% of all QC samples should be between 85.0–115.0\%.  
 10.0\% of all samples can be excluded due to unacceptable internal standard response. |
| Sample dilution      | N≥6  
 CV≤15.0\%  
 Accuracy: 85.0–115.0\% |
| Hemolysis effect     | N≥6  
 CV≤15.0\%  
 Accuracy: 85.0–115.0\% |
| Sample preparation recovery | N≥6  
 Recovery >50.0\% |
| Solution stability   | N≥6  
 CV≤15.0\%  
 Accuracy: 90.0–110.0\% (solution stability)  
 Accuracy: 85.0–115.0\% (stability in plasma) |
| Stability in plasma  | N≥6  
 Carry-over blank in ≥ 2/3 of the samples <30.0\% of the peak area at LLOQ and ≤1/3 of the samples 30.0–50.0\% of the peak area at LLOQ (sample preparation)  
 Carry-over blank internal standard in all samples <5.0\% of the mean peak area of internal standard at CC |

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3.2. Sensitivity (Lower Limit of Quantification)

The lower limit of quantification (LLOQ) was determined by analysing plasma spiked with candesartan at the lowest calibration level (1 ng/mL) independent from the daily calibration curve. The precision (CV) and accuracy in the inter-day runs were within the criteria. The signal to noise ratio at LLOQ was at least 27.5. Figure 2 shows a

Table 2: Precision and accuracy of calibration samples and calibration curve parameters of candesartan in human plasma for sample number 14.

<table>
<thead>
<tr>
<th>Nominal conc. (ng/mL)</th>
<th>Calculated concentration of candesartan (ng/mL)</th>
<th>Slope</th>
<th>Intercept</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.96</td>
<td>0.0107</td>
<td>0.00113</td>
<td>0.9998</td>
</tr>
<tr>
<td>2</td>
<td>5.30</td>
<td>0.0106</td>
<td>0.00122</td>
<td>0.9999</td>
</tr>
<tr>
<td>5</td>
<td>29.3</td>
<td>0.0106</td>
<td>0.00131</td>
<td>1.0000</td>
</tr>
<tr>
<td>10</td>
<td>293.2</td>
<td>0.0107</td>
<td>0.00141</td>
<td>1.0000</td>
</tr>
<tr>
<td>25</td>
<td>293.2</td>
<td>0.0107</td>
<td>0.00141</td>
<td>1.0000</td>
</tr>
<tr>
<td>50</td>
<td>293.2</td>
<td>0.0107</td>
<td>0.00141</td>
<td>1.0000</td>
</tr>
<tr>
<td>100</td>
<td>293.2</td>
<td>0.0107</td>
<td>0.00141</td>
<td>1.0000</td>
</tr>
<tr>
<td>200</td>
<td>293.2</td>
<td>0.0107</td>
<td>0.00141</td>
<td>1.0000</td>
</tr>
<tr>
<td>300</td>
<td>293.2</td>
<td>0.0107</td>
<td>0.00141</td>
<td>1.0000</td>
</tr>
<tr>
<td>400</td>
<td>293.2</td>
<td>0.0107</td>
<td>0.00141</td>
<td>1.0000</td>
</tr>
</tbody>
</table>

Mean (14) 0.99 1.96 2.0 1.5 1.5 1.9 1.7 2.2 1.6

Accuracy (%) 99.0 98.0 99.8 100.4 101.3 100.0 101.2 101.1 99.5 99.5
representative chromatogram of an extracted LLOQ plasma sample. The inter-day precision (CV) of the assay was 8.9% and the accuracy was 95.0%.

3. 3. Linearity

For validation of linearity in the range between 1 and 400 ng/mL blank human plasma was spiked with candesartan at ten concentration levels in seven different days. The calibration curves were constructed by linear regression analysis of the peak-area ratio vs. concentration of candesartan. A weighted linear regression curve \((1/\text{conc.})\) was found to best represent the detector response/concentration relationship for candesartan in human plasma. The calibration function was calculated using Analyst software 1.4.2. Back-calculated values of candesartan in calibration samples and summary of calibration curve parameters are presented in Table 2. The correlation coefficient for 14 repeats of the calibration curve varies between 0.9995 and 1.0000, which shows an excellent linearity of the method.

3. 4. Precision and Accuracy

Intra-day and inter-day precision and accuracy of the method were obtained by an analysis of the replicates of plasma QC samples at four concentration levels (3, 15, 75, 280 ng/mL). The precision of the method was expressed as the coefficient of variation (CV) of the repeated assays and accuracy was given as the ratio between mean found and nominal concentrations. Table 3 shows a summary of the QC data obtained at four concentration levels of QC during fourteen validation runs.

<table>
<thead>
<tr>
<th>Nominal conc. (ng/mL)</th>
<th>Intra-day ((N = 8)) CV (%)</th>
<th>Accuracy (%)</th>
<th>Inter-day ((N = 29)) CV (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2.1</td>
<td>100.3</td>
<td>5.5</td>
<td>100.7</td>
</tr>
<tr>
<td>15</td>
<td>0.6</td>
<td>101.1</td>
<td>1.7</td>
<td>99.9</td>
</tr>
<tr>
<td>75</td>
<td>0.8</td>
<td>101.6</td>
<td>2.1</td>
<td>100.6</td>
</tr>
<tr>
<td>280</td>
<td>1.0</td>
<td>100.2</td>
<td>2.4</td>
<td>100.3</td>
</tr>
</tbody>
</table>

3. 5. Matrix Effect

Samples for the determination of the matrix effect were prepared at low quality control level (3 ng/mL) using eight different sources of human plasma, including one hemolytic and one lipemic plasma sample. Three replicates of each matrix were analyzed in a single run and accuracy for each matrix was calculated. No matrix effect was found in 8 out of 8 different sources of the plasma tested.

3. 6. Matrix Factor

For the matrix factor (MF), extracted blank human plasma samples from eight different human plasma sources (spiked at 3 and 280 ng/mL of candesartan and 100 ng/mL of internal standard) were compared with solutions of candesartan and internal standard at the same concentration levels. The mean MF of candesartan was 1.251 and the mean MF for internal standard was 1.266. The internal standard-normalised MF was also calculated by dividing the MF of candesartan by the MF of the internal standard, and the value 0.988 was obtained \((\text{CV} = 4.1\%)\). According to Viswanathan et al., an absolute matrix factor of about 1 is not necessary for a reliable bioanalytical assay. However, the variability of MF should be tested and fall below 15%. In the present method, the variability in matrix factors is indirectly determined with CV evaluation of the matrix effect. The CV of 24 low concentration QC samples in 8 different sources of plasma is 10.5%, which is less than 15%, so the criterion from Viswanathan et al. is met.

3. 7. Sample Dilution

Dilution of plasma samples was checked at the double value of high QC (560 ng/mL) concentration and five-fold value of 1400 ng/mL (a pool of sample was prepared by spiking drug-free plasma with candesartan). Eight replicates with the dilution factor 2 and eight replicates with the dilution factor 5 were analyzed. No significant deviation in accuracy of candesartan concentration by sample dilution was observed. The dilution accuracy was 98.8\% \((\text{CV} = 0.7\%)\) for the dilution factor 2 and 98.4\% \((\text{CV} = 0.9\%)\) for the dilution factor 5.

3. 8. Sample Preparation Recovery

Extraction recovery of candesartan as well as internal standard was determined at four candesartan QC concentrations (3, 15, 75, 280 ng/mL) and one concentration (100 ng/mL) of internal standard. The recovery of candesartan sample preparation was evaluated by comparing mean candesartan responses of eight processed sources of quality control samples with mean candesartan responses of eight standard solutions of QC samples which were spiked into blank extracts. The CV at each level was less or equal to 8.8\%, with extraction recovery from 63.1 to 74.6%.

3. 9. Hemolysis Effect

Eight replicates of low (3 ng/mL) and high (280 ng/mL) QC samples were prepared with hemolyzed human plasma, processed and analyzed in a single run. Concentrations were calculated to evaluate the hemolysis effect with precision and accuracy. An acceptable accuracy (range 94.7\% to 100.1\%) and precision (range
1.7% to 1.8%) of candesartan concentration was observed. Thus, it was demonstrated that hemolysis did not affect the determination of candesartan in human plasma.

3. 10. Solution Stability

Stability of candesartan as well as internal standard was checked by analyzing eight replicates of stability solutions at different storage conditions in comparison with eight replicates of freshly prepared solutions. Solution stability was checked at two candesartan concentration levels (3 ng/mL and 280 ng/mL) and one concentration level of internal standard (100 ng/mL).

The following storage conditions were tested:
- long-term stock solution stability of candesartan and internal standard for 35 days at +5 °C,
- long-term working solution stability of candesartan and internal standard for 35 days at +5 °C,
- short-term working solution stability of candesartan and internal standard at +23 °C for 24 h.

The prepared solutions for long-term stability were stored for 35 days at a temperature of +5 °C and analyzed only after this period by comparing the peak areas with the freshly prepared solutions. The results are listed in Table 4.

3. 11. Stability in Plasma

The stability of candesartan at different storage conditions in plasma and extracts prepared for the LC-MS/MS analysis was tested at two different concentration levels (3 ng/mL and 280 ng/mL). Samples were stored under different conditions. The mean concentration at each level was compared with the mean of the freshly prepared concentrations.

Stability conditions tested in plasma and extracts (abbreviations are given in the brackets):
- after one freeze-thaw cycle, 24 h at +5 °C and 4 h at room temperature [1x F-T],
- after three freeze-thaw cycles at −20 °C [3x F-T],
- after seven days at −80 °C and two days at −20 °C [−80 °C],
- after freezing at −20 °C for 195 days – long-term stability [195 days],
- 120 h in autosampler at +5 °C – stability of extracted sample [120 h AS].

The results are listed in Table 5.

### Table 4: Stability of candesartan and internal standard in solutions.

<table>
<thead>
<tr>
<th>Solution stability</th>
<th>Conc. (ng/mL)</th>
<th>CV (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long-term stock solution candesartan (35 days, +5 °C)</td>
<td>3</td>
<td>3.4</td>
<td>103.1</td>
</tr>
<tr>
<td></td>
<td>280</td>
<td>0.6</td>
<td>104.6</td>
</tr>
<tr>
<td>Long-term working solution candesartan (35 days, +5 °C)</td>
<td>3</td>
<td>2.7</td>
<td>1077.9</td>
</tr>
<tr>
<td></td>
<td>280</td>
<td>2.4</td>
<td>100.6</td>
</tr>
<tr>
<td>Short-term working solution candesartan (24 hours, +23 °C)</td>
<td>3</td>
<td>4.4</td>
<td>99.3</td>
</tr>
<tr>
<td></td>
<td>280</td>
<td>3.3</td>
<td>100.5</td>
</tr>
<tr>
<td>Long-term stock solution internal standard (35 days, +5 °C)</td>
<td>100</td>
<td>3.0</td>
<td>102.3</td>
</tr>
<tr>
<td>Long-term working solution internal standard (35 days, +5 °C)</td>
<td>100</td>
<td>3.5</td>
<td>100.3</td>
</tr>
<tr>
<td>Short-term working solution internal standard (24 hours, +23 °C)</td>
<td>100</td>
<td>3.0</td>
<td>95.8</td>
</tr>
</tbody>
</table>

### Table 5: Stability of candesartan in human plasma.

<table>
<thead>
<tr>
<th>Stability in plasma</th>
<th>Conc. (ng/mL)</th>
<th>CV (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x F-T</td>
<td>3</td>
<td>3.3</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>280</td>
<td>3.3</td>
<td>99.2</td>
</tr>
<tr>
<td>3x F-T</td>
<td>280</td>
<td>1.2</td>
<td>100.3</td>
</tr>
<tr>
<td>−80 °C</td>
<td>3</td>
<td>6.6</td>
<td>99.1</td>
</tr>
<tr>
<td></td>
<td>280</td>
<td>2.3</td>
<td>99.9</td>
</tr>
<tr>
<td>195 days</td>
<td>3</td>
<td>3.2</td>
<td>99.4</td>
</tr>
<tr>
<td></td>
<td>280</td>
<td>2.3</td>
<td>96.5</td>
</tr>
<tr>
<td>120 h AS</td>
<td>3</td>
<td>1.7</td>
<td>102.7</td>
</tr>
<tr>
<td></td>
<td>280</td>
<td>0.7</td>
<td>101.9</td>
</tr>
</tbody>
</table>

3. 12. Carry-over Test Performance

Determination of the carry-over at the sample preparation step was done by extracting the sample of blank human plasma immediately after the extraction of sample at the highest concentration level of the calibration curve (400 ng/mL) in eight replicates. For all carry-over blank samples the peak area at the retention time of candesartan was below 30% relative to the peak area of the lowest calibration sample at 1 ng/mL of candesartan. The peak area at the retention time of internal standard in all carry-over blank samples was below 5% relative to the peak area of internal standard obtained for the lowest calibration sample.

In order to verify that there is no carry-over in the instrument LC-MS/MS, the mobile phase was injected immediately after eight consequent injections of extract of the highest concentration of the calibration curve. No significant interference was observed at the retention time of candesartan or internal standard in the blank samples. Carry-over in the method is consistent with the acceptance criteria given in Table 1.
3. 13. Application of the Method to Pharmacokinetic Study

The validated method has been successfully used in the bioavailability studies of various candesartan dosage forms, to analyze more than 2500 samples. Tables 6 and 7 show the results for linearity, repeatability and accuracy obtained during one of the studies with the 32 mg candesartan formulation, where the narrowed calibration range 4–400 ng/mL was used.

The method also shows very good precision and accuracy over a longer period of time. A representative pharmacokinetic profile is shown in Figure 3.

4. Conclusions

The article presents validation of LC-MS/MS method for the determination of candesartan in human plasma in accordance with the Viswanathan et al. requirements and OECD Principles of Good Laboratory Practice and its application in pharmacokinetic studies. An advantage of the method was the use of the deuterated candesartan (candesartan-d₄) as the internal standard, which is very similar to candesartan in its physical-chemical properties. With the selected internal standard, most of the unwanted matrix effects were compensated in sample preparation and detection.

Solid-phase extraction was chosen for sample cleanup due to the advantages of selectivity by a clean extract, decreased use of solvents and high efficiency. Besides, there is no need for solvent evaporation. This step was done by extraction on modular Zymark system which enables fast method development with a small number of samples and efficient optimization of each stage of the extraction.

Additional advantage of the method is the fact that due to the small injection volume (10 μL) and clean extract free of interferences, a number of extracts can be analysed on a single chromatographic column.

The validation results indicate that the presented method is sensitive, specific, accurate and reproducible within the wide candesartan concentration range (1 to 400 ng/mL). Therefore, it is suitable for the routine analysis of large numbers of samples in pharmacokinetic studies of different candesartan dosages.

5. References

Povzetek

Za dooloževanje kandesartana v humani plazmi smo razvili in validirali LC-MS/MS metodo in dokazali, da je selektivna, občutljiva in ponovljiva. Za pripravo plazemskih vzorcev smo uporabili ekstrakcijo na trdni fazi. Za kromatografsko separation smo uporabili kolono Gemini C18 z mobilno fazo acetonitril – 5 mM amonijev formiat (90:10, v/v) pri pretoku 0,3 ml/min. Za detekcijo smo uporabili tandemsko masno spektrometrijo v SRM načinu z uporabo pozitivne ionizacije z elektro razprševanjem. Uporabljeni so bili masni prehodi: za kandesartan m/z 443,1 > 263,1 in za kandesartan-d4 kot interni standard 445,5 > 267,1. Po končanem razvoju smo metodo validirali v koncentracijskem območju 1–400 ng/ml v vzorcih humane plazme. Spodnja meja kvantitativne določitve je bila 1 ng/ml. Razvita in validirana metoda se je izkazala za zelo učinkovito pri farmakokinetičnih študijah.