Validation of Metformin Hydrochloride in Human Plasma by HPLC-Photo Diode Array (PDA) for Application of Bioequivalence Study

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Received: March 10, 2011 / Accepted: July 25, 2011 / Published: January 30, 2012.

Abstract: A sensitive and specific high performance liquid chromatography (HPLC) method was developed and validated for the simultaneous determination of metformin hydrochloride (HCl) in human plasma. The HPLC method consists of isocratic elution with a mixture of 60% buffer (10 mM sodium dihydrogenphosphate-10 mM sodium dodecyl sulphate) and 40% acetonitrile with final pH 7.0 with flow rate of 1.0 mL/min on a Kromasil® Akzo Nobel RP-18 (4.6 mm ID × 250 mm, 5 µm) column at an ambient temperature. Photo diode array detection was performed in program mode at 234 nm. The analyte and diazepam as internal standard (IS) were extracted from plasma using 10% trichloroacetic acid. The assay was linear over the therapeutic concentration range of 20-2,500 ng/mL for metformin HCl with correlation coefficient of r = 0.9999. Limit of quantitation was 20 ng/mL. The results obtained for intra/inter day accuracy and precision complied very well with the generally accepted criteria for bio-analytical assay. The method was applied to bioequivalence (BE) study of metformin HCl in healthy Indonesian volunteers after treatment with 750 mg XR metformin HCl. This BE study shows that the two formulations are equivalent so that they were therapeutically interchangeable for each other.

Key words: Metformin HCl, diazepam, HPLC, sodium dodecyl sulphate (SDS), photo diode array (PDA) detector, bioequivalence (BE) study.

1. Introductions

Metformin hydrochloride (N,N-Dimethyl-imido-di-carbonimidic diamide hydrochloride) is a strongly basic bisubstituted guanidine derivative with short side chains. An oral biguanidine antihyperglycaemic agent improves glucose control in patients with type 2 diabetes by lowering both basal and postprandial plasma glucose levels [1-4]. Metformin HCl decreases hepatic glucose production, intestinal absorption of glucose, and improves insulin sensitivity by increasing peripheral glucose uptake and utilization [5-6].

Metformin hydrochloride is slowly and incompletely absorbed from the gastrointestinal tract with a bioavailability of 50% to 60%. Plasma levels peak (C_max) of 1.6 ± 0.38 µg/ml are reached (T_max) at 2.6 ± 0.8 h after oral administration of a single 500 mg dose [2, 6]. It is negligibly bounded to plasma proteins and approximately 90% of the absorbed drug that is eliminated via the renal route within the first 24 h, with plasma elimination half-life of 3.6-6.2 h [5-7].

Metformin hydrochloride is freely soluble in water and it is practically insoluble in acetone, ether, chloroform, and most organic solvents [3], which renders its extraction from aqueous complex plasma matrix difficult [4, 8, 9]. Because metformin HCl is very polar, and is usually analyzed using normal phase with Silica column [4, 8, 9], but it is not economical in time consuming because of complex extraction procedures. Analyzing it using reversed phase (RP) column will not provide optimum result, therefore this method used RP system that is combined with ion-pair. This method used simple sample preparation steps. To
ensure that the used method shall give accurate and reliable result for bioequivalence study, a bioanalytical validation must be performed on the method [10].

The objective of this research is to obtain the validity of metformin analysis method using RP system which is combined with ion-pair in order to be applied for bioequivalence study.

2. Experimental

2.1 Chemicals

Metformin hydrochloride and the internal standard diazepam were obtained from the United State Pharmacopeia reference standard and Menjangan Sakti Company. Sodium dihydrogen phosphate, sodium hydroxide, trichloroacetic acid, sodium dodecyl sulphate, as well as HPLC-grade methanol, acetonitrile were purchased from Merck (Germany) and aquabidestilata from Ikapharmindo (Indonesia). Human plasma was used as the assay blank and for the preparation of spiked plasma standards was obtained from the Indonesian Red Cross (Palang Merah Indonesia, Jakarta).

2.2 Chromatographic Condition

The concentration of metformin HCl in plasma is determined using a high performance liquid chromatography with PDA detection. The HPLC system is Waters 2695 equipped with auto sampler. The separation was performed on Kromasil® C-18, 250 × 4.6 mm, 5 µm column from Akzo Nobel. The wavelength was set at 234 nm. The mobile phase was a mixture of 40% acetonitrile, 0.01 M sodium dodecyl sulphate, 0.01 M sodium dihydrogen phosphate and distilled water to 100%, adjusted to pH 7 at a flow rate of 1 mL/min.

2.3 Matrix Based Standard Solutions and Quality Control Samples

A stock standard solution of metformin (1,000 µg/mL) was prepared by dissolving 10 mg metformin HCl in 10 mL by distilled water. Working standard of metformin was prepared by diluting aliquots of the stock solution with the distilled water to make up final concentration of 100, 25, 10, 2.5, 1, 0.5 and 0.2 µg/mL. These were used to prepare for plasma calibration standard in the linear dynamic range covering 20 to 2,500 ng/mL. Three quality control (QC) plasma samples containing 100, 1,000 and 2,000 ng/mL were prepared. The QC samples were used to determine stability: long term, short term, stock solution, freeze and thaw stability and auto sampler stability. Accuracy and precision were also evaluated using the above QC samples. For the precision was checked by calculating, the variation of the measured value and the accuracy was checked by calculating the difference between the measured values and the actual values. Precision and accuracy should be measured by using minimum of five determinations per concentration and a minimum three concentrations in the range that expected concentration is recommended. The acceptance limit for coefficient of variation (CV) and % differentiation (Diff) is should not exceed ± 15%, except for the lower limit of quantification (LLOQ) where it should not exceed ± 20% of the CV and Diff.

The internal standard diazepam stock standard solution was prepared by dissolving 10 mg in 10 mL methanol to make a final concentration of 1,000 µg/mL.

2.4 Sample Preparation

As much as 600 µL of human plasma which contains metformin hydrochloride will be mixed in a 1.5 mL effendorf vial with 30 µL internal standard (1,000 µg/mL in distilled water) and 600 µL trichloroacetic acid. The sample will be shaken with vortex for 120 seconds and centrifuged at 10,000 rpm for 5 min. After that 1,000 µL supernatant was separated in a clean vial before adding 60 µL of 4 N NaOH. The mixture was vortexed (5 seconds). A 100 µL aliquot of sample was injected into the equilibrated HPLC system.

2.5 Application to Bioequivalence Study

Twelve healthy volunteers were included in this
study. The study protocol was approved by the Ethics Committee of Medical Faculty of University Indonesia and written informed consent was obtained from the volunteers. Metformin was administered in a single dose of 750 mg to the volunteers after overnight fasting. Plasma samples were collected at several intervals after dosing until 30 hours and freeze immediately at -20ºC until assayed.

3. Results and Discussions

3.1 Bioanalytical Validation-Calibration Curves

The standard substance was dissolved in water to make standard stock solution of 1,000 µg/mL metformin HCl. Certain amount of standard stock solution was diluted with blank plasma to make the following concentrations: 0 (blank), zero (blank + internal standard), 20, 50, 100, 250, 500, 1,000 and 2,500 ng/mL, calibration curve was prepared by least square linear regression (y = a + bx), where x was the concentration of metformin HCl, and y was the peak area ratio of metformin HCl to diazepam (internal standard). The standard calibration curve had the following regression equation:

\[ Y = 0.0005 x + 0.0031 \text{ with } r = 0.9999, \text{ as the result of calculation of the slope and intercept.} \]

3.2 Linearity

The linearity of the standard calibration curve was evaluated by calculating the linear correlation coefficient of the curve. The linearity of the standard calibration curve was shown by the linear correlation coefficient (r) of 0.9999.

3.3 Limit Lower of Quantitation (LLOQ)

The lowest concentration of the test substance by which the reliability of assay result can be ensured was determined. The low concentration of the test sample was diluted with blank plasma to 1/2 or 1/4, and the diluted concentrations were measured (in) 5 replicates each within the same determination. The limit of quantitation was the lowest concentration by which the obtained values were within ± 20% of the actual value and the reproducibility was within the CV value 20%. In this research, we observed that the concentration of 101.7 ng/mL gives CV value of 2.69% and % Diff between -6.11% to 1.26%, concentration of 50.85 ng/mL gives CV value of 1.48% and % Diff between -2.37% to 1.37%, and concentration of 20.34 ng/mL gives CV value 2.46% and % Diff 9.77% to 16.86%. Therefore, the limit of quantitation of this procedure was established at 20.34 ng/mL, because the CV and % Diff values are not more than 20%.

3.4 Precision

The precision was checked by calculating the variation of the measured value. The acceptance limit for the precision was within ± 15% for low, medium and high concentrations and within ± 20% for the lower limit of quantification (LLOQ) concentration of the actual value.

3.4.1 Intra-Assay Precision (Within Day Variation)

Three concentrations of the test samples (low, medium, high) were measured in 5 replicates each within the same determination and the CV values were calculated. The CV of the observed values for the three concentrations of the test samples (low, medium, and high) ranged between 1.20-6.91 %, which fulfilled the criteria.

3.4.2 Inter-Assay Precision (Day-to-Day Variation)

Three concentrations of the test samples (low, medium, high) were measured in 5 replicates each in 5 different days and the CV values were calculated. The CV of the observed values for the three concentrations of the test samples were 8.05%, 7.5% and 7.14% for low, medium and high concentration respectively which fulfilled the criteria.

3.5 Accuracy

The accuracy was checked by calculating the difference between the measured values and the actual values. The acceptance limit for the accuracy was within ± 15% for low, medium and high concentrations and within ± 20% for the lower limit of quantification.
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(LLOQ) concentration of the actual value.

3.5.1 Intra-Assay Accuracy (Within Day Variation)

Three concentrations of the test samples (low, medium, high) were measured in 5 replicates each within the same determination and the difference between the measured values and the actual values were calculated. The intra-assay accuracy as shown by the differences between the measured values and the actual values (% diff) for the three concentrations of the test samples ranged between 5.55%-12.95%, 12.28%-3.35%, 12.20%-9.66% for low, medium and high concentrations, respectively, which fulfilled the criteria.

3.5.2 Inter-Assay Accuracy (Day-to-Day Variation)

Three concentrations of the test samples (low, medium, high) were measured in 5 replicates each in 5 different and the difference between the measured values and the actual values were calculated. The inter-assay accuracy as shown by the differences between the measured values and the actual values (% diff) for the three concentrations of the test samples ranged between 12.75%-13.81%, 14.80%-10.00%, 12.20%-6.96% for low, medium and high concentrations respectively, which fulfilled the criteria.

3.5 Selectivity

The LLOQ concentrations of the test samples in 6 different blank samples of human plasma were determined and the CV value was calculated. Blank samples of human plasma were obtained from the Indonesia Red Cross (Palang Merah Indonesia). No endogenous peaks from plasma were found to interfere with the elution of metformin or diazepam as shown at Fig. 1A. The LLOQ concentration of the test samples in six different blank samples of human plasma was determined within the same determination, and the coefficient of variation was 5.44% with % Diff ranged between 4.51%-19.52%.

3.6 Recovery

The recovery absolute and relative was determined using the concentration of the test samples (low, medium, and high). The recovery relative throughout the validation period determined. The relative recovery

![Fig. 1](image-url)  
Representative chromatograms for extracts of plasma samples containing. (A) a blank plasma sample, (B) a spiked sample containing 100 ng/mL metformin (low quality control sample), (C) a spiked sample containing 1,000 ng/mL metformin (medium quality control sample), (D) a spiked sample containing 2,000 ng/mL metformin representing the high quality control sample. All samples were spiked with the 30 µL solution of diazepam 1,000 µg/mL as the internal standard.
for three concentrations of the test samples ranged between 89.87%-113.08% (low), 87.22%-110.00% (medium), 87.80%-106.96% (high). And the absolute recovery for three concentrations of the test samples ranged between 64.25%-86.77% (low), 50.52%-59.77% (medium), and 51.18%-55.98% (high). Absolute recovery was evaluated by measuring the response of processed spike plasma standard expressed as percentage of the responds of pure standard in distilled water.

3.7 Stability

Stability was studied during sample collection storage and processing. All stability studies were conducted using freshly prepared stock solution in the distilled water. Stability experiments extended throughout the analysis duration and until the last test sample was assayed.

3.7.1 Short Term Stability

Using the two concentrations of the test samples (low and high) in plasma, the stability testing was performed under room temperature for 0, 6 and 24 hours. The result of the test determination for short term stability (24 hours at room temperature) was as good as shown by the accuracy value (% Diff) ranged between -2.35%-2.35% (low) and -6.69%(-2.34)% (high). Therefore metformin HCl in plasma was stable for 24 hours at room temperature.

3.7.2 Long Term Stability

Using the two concentrations of the test samples (low and high) in plasma, the stability testing was performed under freezing (-20 °C) storage condition for 0, 7, 40 and 90 days. The result of the test determination for long term stability (90 days at -20 °C) were good as shown by the accuracy value (% diff) ranged between -9.78%-(7.14)% (low) and -12.60%-(9.94)% (high). Therefore metformin HCl in plasma was stable for 90 days at -20°C.

3.7.3 Freeze and Thaw Stability

Using the two concentrations of the test samples (low and high) in plasma, the influence of freeze/thaw cycles (3 cycles, each in duplicates) was investigated. The results of the test determination for three freeze/thaw cycles were good as shown by the accuracy value (% diff) ranged between -11.02%(-6.95)%, -9.18%(-7.57) for low and high concentrations respectively.

3.7.4 Stock Solution Stability

The stability testing was performed under room temperature condition for 6 hours and under refrigerator storage condition for 30 days. The results of the test determination for stock solution stability were good as shown by the accuracy value (% Diff) of -0.44%(-0.42)% for 6 hours at room temperature, and -0.15%-0.14% for 30 days at refrigerator storage condition.

4. Discussion

For validation of bioanalytical methods, the guidance for industry bioanalytical method validation [10] have recommended the accomplishment of accuracy test, precision, specificity, and linearity of the method. Because metformin HCl is very polar, it is usually analyzed using normal phase with Silica column, but it is not economical in terms of cost. Analyzing it using RP column will not provide optimum result, therefore this method use RP system that is combined with ion-pair. In this research SDS as ion-pair was used. The counter-ion in the aqueous mobile phase is used to regulate the retention of metformin, and influence the selectivity and also ensure conditions for efficient chromatographic performance [4, 8].

The IS and analyte were well separated under the described chromatographic condition at retention time of 3.3 and 7 min, respectively. No endogenous component eluted at the retention time of IS and metformin. The total run time was 10 min. Fig. 1 shows the representative chromatograms of blank plasma, plasma sample spiked with metformin at 100 ng/mL, 1,000 ng/mL and 200 ng/mL as QC samples with 30 µL solution diazepam 1,000 ng/mL as the internal standard. The peaks were good shape, completely resolved one
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from another at therapeutic concentrations of metformin. No interference with constituents from the plasma was observed.

The composition of mobile phase is a critical factor for separating metformin and is from endogenous substances. A solution of a mixture of 60% buffer phosphate and 40% acetonitril with final pH 7.0 achieved good resolution and symmetrical peak shapes of the analytes and IS as well as a short run time.

4.1 Assay Validation

The peak area ratio of metformin to IS in human plasma was linear with respect to the analyte concentration over the range of 20-2,500 ng/mL. The LLOQ of metformin was established as 20 ng/mL. The linear regression equation of calibration curve for the analyte was \( y = 0.0005x + 0.0031 \) with \( r = 0.9999 \), where \( y \) was the peak area ratio of the analyte to the IS and \( x \) was the concentration of the analyte. Precision and accuracy of metformin in QC samples fell within the limit acceptability. All values were less then 15%. This validation demonstrated the realiability of our method.

The direct precipitate extraction procedure warranted high sensitivity, good accuracy and precision, relatively high recovery and least time for sample preparation. Metformin HCl is freely soluble in water, and this solvent with trichloroacetic acid was selected as protein-precipitation to deprotein the plasma samples. It was found that the extraction recovery of metformin were 89.87%-113.08% (QC sample low), 87.22%-110.00% (QC sample medium), 87.80%-106.96% (QC sample high) and high enough to fit for quantification. This easy and rapid sample preparation was routinely applied for the bioequivalence study.

Stability result for metformin in plasma are shown in Table 1, indicating that metformin was stable in plasma samples under different storage condition: immediately, after 24 h at ambient temperature, after sample processing and being on the autosampler for 24 h, after three freeze-thaw cycles, and after 90 days stored at -20°C. The stock solutions of metformin and IS were also stable after storage at 2°C for 30 days. This suggests that metformin and IS were stable in experimental condition.

4.2 Bioequivalence Study

The validated method was employed for determination of metformin in human plasma sample collected over 30 hours for bioequivalence study under fasting condition. All 12 volunteers successfully completed the trial according to the protocol. Both metformin HCl formulations were well-tolerated at the administered dose and no serious adverse clinical events were observed. In this study, plots of individual plasma profiles for both formulations and the mean metformin HCl concentration versus time profiles for both formulations are shown in Fig. 3. From the bioequivalence study, the results of pharmacokinetic parameters of \( C_{max} \), \( \text{AUC}_{0-30h} \), \( \text{AUC}_{0-\infty} \), \( T_{max} \) and \( t_{1/2} \) were summarized in Table 2. The parametric 90%

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Stability of metformin concentration in plasma samples.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stability study</td>
<td>Added concentration (ng/mL)</td>
</tr>
<tr>
<td>Short term stability after 24 h at ambient temperature</td>
<td>101.7</td>
</tr>
<tr>
<td></td>
<td>1,017</td>
</tr>
<tr>
<td></td>
<td>2,034</td>
</tr>
<tr>
<td>Three freeze-thaw cycles</td>
<td>101.7</td>
</tr>
<tr>
<td></td>
<td>1,017</td>
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<td></td>
<td>2,034</td>
</tr>
<tr>
<td>Autosampler stability for 24 h</td>
<td>101.7</td>
</tr>
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<td></td>
<td>1,017</td>
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<td></td>
<td>2,034</td>
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<tr>
<td>Stability at -20 °C for 90 days</td>
<td>101.7</td>
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<td>1,017</td>
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<td>2,034</td>
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**Fig. 2** Plasma sample of a human subject, 3 h after the administration of 750 mg XR metformin HCl caplet in bioequivalence study.

**Table 2** Summary the pharmacokinetic parameters of the test and reference products after dosing.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Parameter</th>
<th>Test Parameter</th>
<th>Reference Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$C_{\text{max}}$ (ng/mL)</td>
<td>AUC$_{0-30\text{h}}$ (ng.hr/mL)</td>
<td>AUC$_{0-\infty}$ (ng.hr/mL)</td>
</tr>
<tr>
<td>Test</td>
<td>1,268.92 ± 215.43</td>
<td>9,708.32 ± 2,446.07</td>
<td>10,085.31 ± 2,451.52</td>
</tr>
<tr>
<td>Reference</td>
<td>1,207.07 ± 244.21</td>
<td>9,395.80 ± 1,753.09</td>
<td>9,804.09 ± 1,720.35</td>
</tr>
</tbody>
</table>

Confidence interval on the mean of the difference (test-reference) between log-transformed values of the two formulations were 97.00% to 115.98%, 94.78% to 109.50% and 93.77% to 109.87% for $C_{\text{max}}$, AUC$_{0-30\text{h}}$ and AUC$_{0-\infty}$, respectively. The results indicate that the two formulations can be considered equivalent in the extent of absorption and can be used interchangeably.

A highly sensitive and specific HPLC method for the
determination of metformin HCl in human plasma has been developed and validated, with a lower quantitation limit 20 ng/mL. Validation experiments have shown that the assay has good precision and accuracy over a wide concentration range (20-2,500 ng/mL). This BE study shows that the two formulations are equivalent so that they can therapeutically interchangeable for each other.

Acknowledgments

The authors thank to Ferron Par Pharmaceutical Company, Indonesia for financial support of the project.

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