Determination of Metformin in Human Plasma Using Normal Phase High Performance Liquid Chromatography

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A simple, selective, sensitive, accurate, and precise normal phase HPLC method coupled with UV detection has been developed and validated for the determination of metformin in human plasma. After protein precipitation with acetonitrile, metformin was extracted with dichloromethane. The mobile phase consisted of acetonitrile and phosphate buffer (0.05M) (60:40 % v/v) pH 7.0, the stationary phase was a normal phase silica column (250×4.6 mm ID, 5 μm particle size). Detection was carried out using a UV detector set at 235 nm. The method was linear over the concentration range 0.016-2.709 μg/ml (y = 0.7898X + 0.0048) and gave a limit of quantitation of 16 ng/ml. Analytical recovery, measured over three days, averaged 94.88%. The interday precision ranged 3.2 to 11.8 CV (%) for four quality control samples including LLOQ, low, medium, and high. Metformin was found to be stable in plasma and in working standard solutions during sample collection, storage, and processing as well as in five freeze thaw cycles. The described HPLC method was successfully employed for the analysis of authentic samples collected from three bioequivalence studies involving 32 volunteers each. The average concentration – time profiles were plotted from the three bioequivalence studies which involved three doses of 500 mg/tablet, 850 mg/tablet and 1000 mg/tablet under fasting conditions. Slow GI absorption and linear pharmacokinetics characterized the disposition of metformin.

Key words: metformin, HPLC, normal phase, bioequivalence

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Introduction

Metformin hydrochloride (N,N-Dimethyl-imidodi-carbonimidic diamide hydrochloride) is a strongly basic bisubstituted guanidine derivative with short side chains. It is an orally administered biguanide that has been widely used in the treatment and management of non-insulin dependent diabetes mellitus (NIDDM). Metformin lowers both basal and postprandial elevated blood glucose in patients with NIDDM; when hyperglycemia cannot be satisfactorily managed on diet alone (1-4). The highly polar compound escapes metabolism almost entirely, and is eliminated via renal excretion (5,6). As shown below, metformin exists in two tautomeric forms in acidic media. Metformin is practically insoluble in most organic solvents (7), which renders its extraction from the aqueous complex plasma matrix difficult (8,9).

Structure of Metformin Hydrochloride

Several chromatographic methods have been suggested for the determination of metformin in human plasma. Among others, these have included capillary electrophoresis (9) or expensive techniques such as HPLC-MS/MS (10-14) or techniques which necessitated derivatization prior to separation such as GC coupled with ECD (15,16). Among the different HPLC techniques used including ion-exchange, ion-pair and reversed-phase, the most successful was the use of normal phase HPLC coupled with UV detection (5,8,9,17-24). Previously described methods suffered from several disadvantages including use of complex extraction procedures which were tedious and time consuming. Ultra-filtration and column-switching techniques have been suggested to improve specificity and selectivity (19). Attempts to reproduce the performance of some published methods, which extracted metformin from alkaline solutions using liquid-liquid extraction followed by back extraction with acetic acid failed to demonstrate the required specificity (24).

For pharmacokinetic, bioequivalence, and therapeutic drug monitoring studies; a selective, sensitive, accurate, and precise method is required. The present work describes a newly developed HPLC method coupled with UV detection which utilized silica stationary phase in combination with an optimized mobile phase. Contrary to previous methods, the new liquid liquid extraction technique which preserved the plasma pH. The method demonstrated high selectivity and specificity without compromising precision and accuracy. The applicability of the method was demonstrated in three bioequivalence studies which investigated three metformin doses of 500, 850 and 1000mg/tablet for the first, second, and third biostudy respectively.

Experimental

Materials:
Metformin hydrochloride (99.13%) and the internal standard, bisoprolol (99.9%), were obtained from the Pharmaceutical Research Unit, Royal Scientific Society, Amman, Jordan. Potassium dihydrogen phosphate, potassium hydroxide, as well as HPLC-grade methanol, acetonitrile, and dichloromethane were purchased from Merck (Germany). Human plasma used as the assay blank, and for the preparation of spiked plasma standards was obtained from the Jordan Blood Bank (Ministry of Health, Amman, Jordan). HPLC grade water was prepared at the PRU by further distilling and deionizing water after initiating two reversed-osmosis cycles. An Elgacan column (C114) was employed for deionization.

Instrumentation and Software:
The HPLC system consisted of a solvent delivery system (Spectra Physics P2000), a Rheodyne valve and syringe type loading sample injector (Model 7125) (100 μl sample loop), a normal phase silica guard column (5.0X4.5mm) and silica column (250X4.6 (mm) ID, 5μm particle size, Hichrom LTD, England). The detector was a UV/Visible detector (Spectra System UV 1000) fixed at 235 nm. Data was analyzed by Chromquest software version 2.51 running under MS windows 98.
The mobile phase consisted of acetonitrile and a 0.05 M potassium phosphate buffer (60:40% (v/v)). The pH was adjusted to 7.0 using potassium hydroxide (5.0 M). The mobile phase was filtered prior to use using a 0.45µm filter. The flow rate was set at 1.0 ml/min, and the system was operated at ambient temperature.

Matrix based standard solutions and quality control samples:
A stock standard solution of metformin (1083.476 µg/ml) was prepared by dissolving 34.99 mg metformin HCl in 25 ml of the mobile phase. Working standards of metformin were prepared by diluting aliquots of the stock solution with the mobile phase to make up final concentrations of 108.39 and 10.85 µg/ml. These were used to prepare plasma calibration standards in the linear dynamic range covering 0.016 to 2.706 µg/ml. Three quality control (QC) plasma samples containing 0.016, 0.049, and 1.354 µg/ml were prepared. The QC samples were used to determine stability: long term, short term, stock solution, freeze-thaw stability and autosampler stability. Accuracy and precision were also evaluated using the above QC samples.

The internal standard bisoprolol stock standard solution was prepared by dissolving 21.43 mg bisoprolol fumarate in a 25 ml aliquot of mobile phase to make a final concentration of 27.558 µg/ml. 1.0 ml of each plasma sample was spiked with an aliquot of bisoprolol IS solution to make up a final concentration of 8.003 µg/ml IS. Prior to extraction, each sample was vortexed (30 sec) to ensure omogenous mixing of bisoprolol. All plasma samples were stored frozen at -86°C until assay.

Method Development

As expected, the use of RP-HPLC failed because of the high polarity of metformin. Although both the cyano and C8 bonded phases demonstrated reasonable retention times (4.2 min), specificity was much higher when a silica stationary phase was employed. A retention time, which averaged 8.2 minutes, was obtained after optimizing a mobile phase containing acetonitrile and a 50mM potassium dihydrogen phosphate buffer (60:40 v/v (%), pH 7.0). Increasing the aqueous composition of the mobile phase to values higher than 45% resulted in broad peaks which adversely affected the sensitivity. All reported extraction procedures have been investigated; however, lack of specificity and/or low analytical recovery prohibited their use. This includes a recently reported method which involved extraction with a mixture of 1-butanol and n-hexane 50:50 v/v (%) from an alkaline plasma. The following solvents have been investigated: chloroform, ethylacetate, diethyl ether, cyclohexane, and n-hexane. Extraction with dichloromethane resulted in high specificity and high recovery without compromising precision and accuracy.

Sample Extraction:
To a 1.0 ml plasma aliquot, 1.5 ml aliquot of acetonitrile was added. The mixture was vortexed (30 sec) and centrifuged (3500 rpm for 8.5 min), the supernatant was then separated and introduced into a clean test tube. A 5.0 ml portion of dichloromethane was added and shaken (5.0 min) before centrifugation (4000 rpm, 5.0 min). 400 µl aliquot of the aqueous layer was separated in a clean vial before adding 10 µl of 1.0 M HCl. The mixture was vortexed (5 sec). A 100 µl aliquot of the sample was injected onto the equilibrated HPLC system.

Results and Discussion

1. Validation:
1.1. Recovery:
Absolute recovery was evaluated by measuring the response of processed spiked plasma standards expressed as percentage of the response of pure standards in the mobile phase. Figure 1 (A,B, and C) illustrates representative chromatograms for: (A) a blank extract together with a plasma extracts for a low quality control sample (0.049µg/ml) (B) an extract of a medium quality control sample (1.354 µg/ml), and (C) an extract of a high quality control sample (2.164µg/ml). Figure 2 illustrates area ratios of standard solutions in the mobile phase compared with those extracted from the plasma matrix.

The average absolute recoveries for quality control samples containing 0.016, 0.049, 1.354, and 2.164 µg/ml metformin levels, measured over three days, were 63.48%, 65.83%, 64.56%, and 64.39%. Analytical recoveries of metformin, measured over three days, were also evaluated for the same concentrations. These averaged 91.67%, 97.28%, 93.60%, and 96.97% for the lower limit of quantitation, the low, the medium and the high quality control samples respectively. The within-day analytical recoveries were: 93.75%, 97.96%, 93.57% and 95.95% respectively.
DETERMINATION OF METFORMIN IN HUMAN PLASMA

Figure 1. Representative chromatograms for extracts of plasma samples containing (A) a blank plasma sample and a spiked sample containing metformin at the lower limit of quantitation (0.016 µg/ml) (B) a spiked sample containing 1.353 µg/ml metformin (medium quality control sample) (C) a spiked sample containing 2.165 µg/ml metformin representing the high quality control sample. All samples were spiked with the 8.003 µg/ml bisoprolol as the internal standard.

The authors recognize that estimation of recovery is an area where practice differs among analytical chemists, and there is no single well defined approach to estimating, expressing and applying recovery information. The present work suggests that the use of analytical recovery, in principle, eliminates negative determinate errors due to bias pertaining to loss of the analyte during sample processing. The results demonstrate that an average analytical within-day recoveries of 94.88% was obtained.

![Area Ratio](image)

**Figure 2.** Area ratios (area of metformin / area of bisoprolol) versus concentrations of metformin using (A) Extracts of matrix based calibrators, (B) metformin standard solutions in mobile phase.

1.2. Linearity:

A series of standard plasma solutions, previously spiked with metformin, were employed as matrix standards for constructing the calibration curve. The analytes: metformin and the internal standard bisoprolol were extracted using the method described above. The matrix based calibration curve was linear over the concentration range 0.016-2.709 µg/ml ($y = 0.7898x + 0.0048$, $r^2=0.999$). The standard deviation of $y$ regressing on $x$ ($S_{y/x}$) was 0.0256, $S_y$ 0.0328 and $S_x$ 0.0015. The number of theoretical plates $N$ was 787 for metformin, resulting in a height equivalent to a theoretical plate of 0.32 mm.

1.3. Sensitivity:

Two types of sensitivities were calculated: (a) calibration sensitivity, which was equated with the slope of the calibration graphs, and (b) analytical sensitivity ($\gamma$), which accounted for variations in the standard deviation of the analytical signals measured for different concentration levels of metformin. The calculated calibration sensitivity was 0.7898 ($\pm0.0528$), whereas the analytical sensitivities are summarized in table 1.
Table 1: Analytical sensitivities of four quality control samples.

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>S (±)</th>
<th>Analytical sensitivity (γ) (Slope/S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLOQ</td>
<td>0.016</td>
<td>0.002</td>
</tr>
<tr>
<td>QCL</td>
<td>0.049</td>
<td>0.005</td>
</tr>
<tr>
<td>QCM</td>
<td>1.354</td>
<td>0.072</td>
</tr>
<tr>
<td>QCH</td>
<td>2.167</td>
<td>0.095</td>
</tr>
</tbody>
</table>

LLOQ : lower limit of quantitation
QCL : low quality control sample
QCM : medium quality control sample
QCH : high quality control sample

1.4. Precision and Accuracy:
Ten replicate injections of the same standard mixture in the mobile phase, containing four different concentrations of metformin, each mixed with the internal standard bisoprolol (8.003 μg/ml) were employed to determine instrument precision. Six replicate measurements of each quality control matrix based standards were chromatographed to evaluate method precision. Intra-day precision was also evaluated by analyzing plasma samples containing four different concentrations. Inter-day precision was evaluated for the same concentrations fourteen times over a period of two months. The results are summarized in table 2.

Table 2: Summary of precision data including instrument, method, inter and intraday precision.

<table>
<thead>
<tr>
<th>Metformin Concentration(μg/ml)</th>
<th>Instrument precision CV (%)</th>
<th>Method Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intraday precision CV (%)</td>
<td>Interday Precision CV (%) (14 days)</td>
</tr>
<tr>
<td>0.016</td>
<td>2.4</td>
<td>12.5</td>
</tr>
<tr>
<td>0.049</td>
<td>2.0</td>
<td>7.8</td>
</tr>
<tr>
<td>1.354</td>
<td>1.0</td>
<td>3.9</td>
</tr>
<tr>
<td>2.167</td>
<td>0.8</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Individually processed plasma quality control samples including 0.016, 0.049, 1.354, and 2.167 μg/ml were prepared and analyzed as described previously. The relative error (%) were calculated and presented in table 3.

Table 3: Summary of absolute error (bias) and relative error (accuracy) for four quality control matrix based samples.

<table>
<thead>
<tr>
<th>Accuracy</th>
<th>Analytical Concentration (μg/ml)</th>
<th>Measured Concentration (μg/ml)</th>
<th>Error (Bias)</th>
<th>Relative Error (%)</th>
<th>Confidence Interval (95%) (Confidence level)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.016</td>
<td>0.017</td>
<td>0.001</td>
<td>5.88</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>0.049</td>
<td>0.049</td>
<td>0.000</td>
<td>0.00</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>1.354</td>
<td>1.254</td>
<td>-0.101</td>
<td>7.32</td>
<td>0.112</td>
</tr>
<tr>
<td></td>
<td>2.167</td>
<td>2.082</td>
<td>-0.084</td>
<td>3.81</td>
<td>0.152</td>
</tr>
</tbody>
</table>

1.5. Specificity and Selectivity:
Specificity was tested against endogenous compounds and against potential interferences. To determine specificity with respect to endogenous compounds, six blank plasma samples, collected under controlled conditions, were extracted and chromatographed. The responses at the retention times of metformin and bisoprolol were compared with those obtained from an extract of a previously spiked plasma sample at the LLOQ (0.016 μg/ml) of metformin with 8.003 μg/ml bisoprolol. No interferences were detected at the retention times of either metformin or the internal standard. In order to prove that the matrix has no influence on the analytical signal, potential interferences from caffeine and common OTC drugs including: acetaminophen, theophylline, ibuprofen were investigated. None of the investigated interferents showed analytical signal(s) at the retention times of either metformin or the internal standard.

1.6. Stability:
Stability was studied during sample collection, storage, and processing. All stability studies were conducted using freshly prepared stock solutions in the mobile phase.
Stability experiments extended throughout the analysis duration and until the last test sample was assayed. Stability studies investigated metformin (0.049 μg/ml, 2.167 μg/ml) and bisoprolol (8.003 μg/ml). In order to determine short-term matrix solution stability, samples were thawed once, and kept at room temperature for 6, 12, and 24 hours.
prior to their extraction and consequent analysis. Long term matrix based solution stability was evaluated under prolonged storage conditions (-86°C) during the study period. Freeze and thaw stability covered five freeze-thaw cycles. Both metformin and bisoprolol were stable under the experimental conditions.

2. Application of the developed method:

The described method was employed for the determination of metformin in authentic plasma samples collected over 32 hours for three bioequivalence studies under fasting conditions, these involved dosing with (1) 500mg metformin/tablet, (2) 850 mg metformin/tablet, and (3)1000 mg metformin /tablet. The concentration-time profiles for the average metformin levels from 32 participants who completed each of the three bioequivalence studies for the reference and test products are illustrated in figure 3 (A, B, and C). For each study, the pharmacokinetic parameters of $C_{\text{max}}$, $T_{\text{max}}$, $AUC_{0-\text{last}}$, $AUC_{0-\infty}$, and $t_{1/2}$ were summarized in table 4 (A, B, and C).

Table 4: Summary of the pharmacokinetic parameters of the test and reference products after dosing with

(A) 500mg metformin / tablet

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Test</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ ($\mu$g/ml)</td>
<td>1.12 (±0.29)</td>
<td>1.09 (±0.29)</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (hr)</td>
<td>2.69 (±0.73)</td>
<td>2.29 (±0.76)</td>
</tr>
<tr>
<td>$t_{1/2}$ (hr)</td>
<td>4.35 (±1.47)</td>
<td>4.57 (±1.58)</td>
</tr>
<tr>
<td>$AUC_{0-\text{last}}$ ($\mu$g.hr/ml)</td>
<td>7.22 (±1.54)</td>
<td>6.99 (±1.71)</td>
</tr>
<tr>
<td>$AUC_{0-\infty}$ ($\mu$g.hr/ml)</td>
<td>7.41 (±1.58)</td>
<td>7.16 (±1.72)</td>
</tr>
</tbody>
</table>

(B) 850mg metformin / tablet

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Test</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ ($\mu$g/ml)</td>
<td>1.53 (±0.43)</td>
<td>1.38 (±0.45)</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (hr)</td>
<td>2.77 (±0.93)</td>
<td>3.15 (±0.97)</td>
</tr>
<tr>
<td>$t_{1/2}$ (hr)</td>
<td>5.06 (±1.79)</td>
<td>5.04 (±2.50)</td>
</tr>
<tr>
<td>$AUC_{0-\text{last}}$ ($\mu$g.hr/ml)</td>
<td>9.44 (±2.23)</td>
<td>9.27 (±2.21)</td>
</tr>
<tr>
<td>$AUC_{0-\infty}$ ($\mu$g.hr/ml)</td>
<td>9.65 (±2.20)</td>
<td>9.58 (±2.22)</td>
</tr>
</tbody>
</table>

(C) 1000mg metformin / tablet

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Test</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ ($\mu$g/ml)</td>
<td>1.69 (±0.41)</td>
<td>1.68 (±0.47)</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (hr)</td>
<td>2.74 (±1.04)</td>
<td>2.43 (±0.68)</td>
</tr>
<tr>
<td>$t_{1/2}$ (hr)</td>
<td>6.12 (±2.97)</td>
<td>5.20 (±2.97)</td>
</tr>
<tr>
<td>$AUC_{0-\text{last}}$ ($\mu$g.hr/ml)</td>
<td>10.14 (±2.94)</td>
<td>9.68 (±2.89)</td>
</tr>
<tr>
<td>$AUC_{0-\infty}$ ($\mu$g.hr/ml)</td>
<td>10.48 (±3.05)</td>
<td>9.95 (±2.93)</td>
</tr>
</tbody>
</table>

Figure 3. Mean plasma concentrations versus time (hr) profiles for 26 volunteers dosed with the test drug product (A) after an oral dose of 500 mg metformin/tablet, (B) after an oral dose of 850 mg metformin/tablet (C) after an oral dose of 1000 mg metformin / tablet
As illustrated in figure 3 and summarized in table 4, the pharmacokinetic parameter $T_{\text{max}}$, which averaged 2.73 hr for the test product and 2.62 hr for the reference product, demonstrated that the GI absorption is relatively slow. Furthermore, plots of $\text{AUC}_{0\rightarrow\infty}$ vs dose as well as those for $C_{\text{max}}$ vs dose demonstrated that metformin pharmacokinetics were linear with $R^2$ value of 0.9996, 0.945, 0.999, and 0.9699 for $C_{\text{max}}$(test), $C_{\text{max}}$(reference), $\text{AUC}_{0\rightarrow\infty}$(test), and $\text{AUC}_{0\rightarrow\infty}$ (reference) values respectively. Disposition was not dose dependent probably due to saturable active absorption.

**Conclusion**

A selective, sensitive, precise and accurate method has been developed for the analysis of samples after dosing with either 500mg, 850mg, or 1000 mg metformin tablets under fasting conditions. A limit of quantitation of 16 ng/ml enabled an accurate evaluation of metformin pharmacokinetic parameters. The method has been successfully employed for the determination of concentration-time profiles of metformin in three bioequivalence studies.

**References**