A Novel Extractionless HPLC Fluorescence Method for the Determination of Glyburide in the Human Plasma: Application to a Bioequivalence Study


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Abstract Purpose. To develop a simple, sensitive and rapid HPLC fluorescence method with single step sample preparation for the determination of glyburide in the human plasma. Methods. Glyburide and ketoconazole (internal standard) were extracted from the 0.5 mL plasma by addition of 0.5 mL acetonitrile and 50 µL CuSO₄ solution (5% w/v in water). The separation was achieved on the Kingsorb 3 µm, C8 reverse phase column at ambient temperature with a mobile phase consisted of 45% buffer solution (0.05 M NH₄H₂PO₄), 40% acetonitrile and 15% methanol adjusted to pH 5.7 by diluted ammonia solution. A fluorescence detector was set at 235 nm excitation wavelength and 354 nm emission wavelengths to monitor eluted components. Results. The internal standard and glyburide eluted at about 6.7 and 9.6 min, respectively at the flow rate of 1 mL/min. The regression equation was established for every calibration curves (5 ng/mL to 400 ng/mL), which resulted in the correlation coefficient of 0.99 or greater. The absolute recovery ranged from 94.32 to 98.12% and the relative recovery ranged from 91.12 to 97.15%. The intraday coefficient of variation (CV) ranged from of 6.52 to 12.35% and interday varied from 6.21 to 16.07%. The limit of quantitation (LOQ) of glyburide was set to five ng/mL. Conclusion. This simple, rapid and sensitive method is suitable for pharmacokinetic, bioavailability and bioequivalence studies.

INTRODUCTION

The sulphonylurea hypoglycemic agent glyburide (Figure 1) also referred as glibenclamide in British pharmacopoeia is widely used in the treatment of type 2 diabetes mellitus. The usual initial dose for this highly potent drug is 2.5 to 5 mg daily given as a single dose just before the breakfast (1). Because of relatively pro-longed duration of action and episodes of hypoglycemia associated with the treatment, it is essential to monitor the bioavailability of glyburide (2). Several high performance liquid chromatographic methods with UV or fluorescence detector have been developed for the determination of glyburide in body fluids (3-13). Most of the methods are utilizing liquid-liquid extraction or solid phase extraction of drug. All these methods require lengthy sample processing and are time consuming.

Figure 1: Chemical structure of glyburide and ketoconazole (IS)

This paper describes a simple HPLC with fluorescence detection for the determination of glyburide following precipitation of proteins. The sensitivity and easy sample processing make it suitable for monitoring the composition of biological samples such as plasma and serum. This method is enough sensitive (5 ng/mL) to
monitor the biological samples obtained for pharmaco-
kinetics, bioavailability and therapeutic drug monitor-
ing.

**MATERIALS AND METHODS**

**Materials**

The master reference standard of glyburide and keto-
conazole was purchased from USP (USP, 12601 Twin-
brook Parkway, Rockwille, MD 20852, USA). Subsequently, pharmaceutical grade raw material of
glyburide and ketoconazole was evaluated against the
master reference standard and considered as reference
standards. All other chemicals and reagents were of
analytical grade (E.Merck, Darmstadt, Germany). The
solvents used were of HPLC grade purchased from
Carlo-Erba, Milan, Italy. The deionized water was pre-
pared using Milli-Q system (Millipore, Molsheim,
France)

**HPLC conditions**

The chromatographic system was composed of a sol-
vent delivery pump (model 600), an auto injector
(model 717), a scanning fluorescence detector (model
474) with excitation and emission wavelengths set at
235 nm and 354 nm, respectively; all from Waters asso-
ciates (Milford, MA, U.S.A). A 15 cm x 4.6 mm i.d.
Kingsorb C8 analytical column packed with 3 µm par-
ticle size and a precolumn insert packed with C8 (Phe-
nomenex, Torrance, CA, U.S.A.) were used for
separation. The mobile phase consisted of 45% buffer
solution (0.05 M NH₄H₂PO₄), 40% acetonitrile and
15% methanol adjusted to pH 5.7 by diluted ammonia
solution was pumped at 1 mL/min flow rate. The
mobile phase was prepared daily, filtered through 0.45
µm nylon membrane filter (Whatman, Maidstone,
England) and degassed before use. The chromatograms
were acquired and analyzed using EZ-Chrom chroma-
tography data system (Shimadzu, Columbia, MD,
U.S.A.). All work was carried out at 22°C laboratory
temperature.

**Stock solutions**

Accurately weighed 50 mg glyburide reference stan-
dard was dissolved and diluted to 200 mL in acetonitrile
to give primary standard at the concentration of 250
µg/mL. Subsequent dilutions were made to give 0.1,
0.2, 0.5, 1, 2, 4 and 8 µg/mL stock solutions for calibra-
tion curve and 0.3, 1.5 and 6 µg/mL stock solutions for
validation samples. The primary standard of ketocona-
zole (IS) was prepared at 1 mg/mL by dissolving accu-
rateley weighed 100 mg ketoconazole reference standard
to 100 mL in acetonitrile. Further dilution was made to
obtain stock solution of internal standard at the con-
centration of six µg/mL. Primary standards and stock
solutions were prepared once weekly. All the solutions
were stored at (8 to 12°C).

**Calibration samples**

In a series of 12 mL, PTFE sealed glass test tubes con-
taining 0.5 mL blank plasma and 25 µL of 6 µg/mL
ketoconazole (internal standard) were added with 25
µL stock solution of appropriate concentration to pro-
vide calibration samples of 5, 10, 25, 50, 100, 200 and
400 ng/mL. After brief vortex mixing 50 µL of CuSO₄
(5% solution in water) and 500 µL of acetonitrile were
added to precipitate proteins. Each calibration sample
was then vortex–mixed and centrifuged for 15 min.
The supernatant solution was transferred to an auto
sampler vial (1 mL capacity) and 25 µL aliquot was
injected in to the HPLC column.

**Plasma samples**

Samples obtained from the subject were processed simi-
lar to calibration sample as mention under calibration
samples except that 25 µL acetonitrile was added in
place of 25 µL stock solution.

**Pharmacokinetics**

The study protocol was approved by the Internal
Review Board at the Prince Fahd Bin Sultan Hospital,
Tabuk, Saudi Arabia. Twenty six healthy male subjects
participated in a single dose fasting crossover bioequiva-
ience study. Following informed consent each subject
received 5 mg glyburide tablet either of test formula-
tion (Gliburan, batch no. PD27, manufactured by
Tabuk Pharmaceutical Mfg Co., Saudi Arabia) or re-
ference formulation (Daonil, batch no. 41C224, manu-
factured by Hoechst AG, Germany). Blood samples (6
mL) were collected in heparinized tubes at predose and
0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, 8.0,
9.0, 10.0, 12.0, 16.0, and 24 h following oral administra-
tion of the drug. Plasma was immediately separated by
centrifugation and stored at –50°C in 5 mL polypropy-
lene tubes until analyzed.
RESULTS AND DISCUSSIONS

The mobile phase used for the assay provided a well defined separation between the drug, internal standard and endogenous components (Figure 2). The optimum flow rate for the mobile phase (1 mL/min) resulted in an analysis time of less than 15 min/sample. Possible interference from other drugs was investigated by examining their peak separation from glyburide using the same HPLC conditions. All the investigated OTC drugs including paracetamol, caffeine, aspirin and ibuprofen showed no interference in the assay. The retention times for ketoconazole (internal standard) and glyburide were 6.7 and 9.6 minutes, respectively. The zero hour (predose) samples of all subjects showed no interference at retention time of both the drugs. The possible interference from the metabolites (cis-3-hydroxyglyburide and trans-4-hydroxyglyburide) was assessed by running a set of samples from a subject without addition of internal standard that gave clear base line around the retention time of internal standard. Theoretically, hydroxy metabolites are relatively more polar than parent drug and they should elute before the peak of glyburide in reversed phase chromatography system.

The quantification of the chromatogram was performed using peak area ratios of the drug to internal standard. The calibration curves were constructed routinely for spiked plasma containing 5 ng/mL, 10 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL, 200 ng/mL and 400 ng/mL of glyburide during the process of prestudy validation and in-study validation. Least squares linear regression analysis of the calibration curves resulted in the following equations: $Y = 0.0063X + 0.0374$, $R^2 = 0.998$. Standard curves of glyburide in plasma were constructed on eleven different days over a four weeks period to determine the variability of the slopes and intercepts. The results indicated little interday variability of slopes and intercepts, as well as good linearity ($r^2>0.99$) over the concentration range studied. The coefficient of variations for the slopes was 6.58%, which indicates good precision of the method. The lowest standard on the calibration curve was five ng/mL and that was accepted as LOQ on the basis of less than 20% CV as acceptance criteria.

The intraday precision was evaluated by analysis of plasma samples containing glyburide at three different concentrations i.e. 5 ng/mL, 75 ng/mL and 300 ng/mL using six replicate determinations for three occasions. The intraday precision showed a coefficient of variation (CV) of 6.52 to 12.35% (Table 1). The interday precision was similarly evaluated over two-week period and showed a CV varying from 6.21 to 16.07% (Table 1).

The absolute recovery of glyburide was determined by comparing the peak area of the drug obtained from the plasma with peak area obtained by the direct injection of pure aqueous drug standard at three different concentrations on three occasions. The relative recovery of the drug was calculated by comparing the concentration obtained from the drug supplemented plasma to the actually added concentration. As shown in the Table 2, absolute recoveries of glyburide ranged from 94.32 to 98.12% and the relative recovery ranged from 91.12 to 97.15%.

![Figure 2: Representative chromatograms of: A) blank plasma, B) plasma spiked with 150 ng/mL ketoconazole (IS) and 100 ng/mL glyburide and, C) plasma sample from a subject 2 h after administration of 5 mg glyburide tablet, the concentration of glyburide is 51.7 ng/mL.](image)
The stability studies of plasma samples spiked with glyburide (5 ng/mL, 75 ng/mL and 300 ng/mL) were performed over four weeks (Table 3). In addition, stability studies of plasma samples spiked with glyburide were performed for 24 hour period at room temperature storage and for three freeze-thaw cycles. The plasma samples were stored in the freezer at -50°C until the time of analysis. The results show that glyburide can be stored frozen in the plasma for one month without degradation. The results of short term storage at room temperature, solution stability and freeze-thaw cycles indicated no degradation of glyburide in plasma as well as in solution therefore samples could be handled without special precautions.

### Table 2: Recovery of glyburide and ketoconazole (IS) in human plasma

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Peak area in plasma</th>
<th>Peak area in mobile phase</th>
<th>Recovery %</th>
<th>Relative recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 ng/mL glyburide</td>
<td>38344 (9.58)</td>
<td>40653 (2.32)</td>
<td>94.32</td>
<td>93.94± 6.82</td>
</tr>
<tr>
<td>75 ng/mL glyburide</td>
<td>162803 (8.49)</td>
<td>166926 (0.97)</td>
<td>97.53</td>
<td>91.12±7.12</td>
</tr>
<tr>
<td>300 ng/mL glyburide</td>
<td>602227 (5.54)</td>
<td>613766 (1.88)</td>
<td>98.12</td>
<td>97.15±4.31</td>
</tr>
<tr>
<td>150 ng/mL ketoconazole</td>
<td>327065 (6.55)</td>
<td>336556 (0.92)</td>
<td>97.18</td>
<td>95.27±6.13</td>
</tr>
</tbody>
</table>

*Values in parentheses are % CV*

### Table 3: Stability of glyburide in human plasma during storage and sample handling

<table>
<thead>
<tr>
<th>Concentration in plasma</th>
<th>Freeze and thaw:</th>
<th>Short term (plasma sample at room temperature):</th>
<th>Long term (plasma sample at -50°C):</th>
<th>Auto sampler (extracted sample at room temperature):</th>
<th>Stock solution (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cycle 1</td>
<td>Cycle 2</td>
<td>Cycle 3</td>
<td>After 2 h</td>
<td>After 8 hr</td>
</tr>
<tr>
<td>15 ng/mL glyburide</td>
<td>15.5</td>
<td>15.1</td>
<td>14.2</td>
<td>15.11</td>
<td>15.73</td>
</tr>
<tr>
<td>75 ng/mL glyburide</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>300 ng/mL glyburide</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The stability studies of plasma samples spiked with glyburide (5 ng/mL, 75 ng/mL and 300 ng/mL) were performed over four weeks (Table 3). In addition, stability studies of plasma samples spiked with glyburide were performed for 24 hour period at room temperature storage and for three freeze-thaw cycles. The plasma samples were stored in the freezer at -50°C until the time of analysis. The results show that glyburide can be stored frozen in the plasma for one month without degradation. The results of short term storage at room temperature, solution stability and freeze-thaw cycles indicated no degradation of glyburide in plasma as well as in solution therefore samples could be handled without special precautions.
The bioequivalency of two glyburide products, reference and generic formulation was compared in 26 healthy male volunteers who received a single 5 mg oral dose in crossover design. As shown in (Figure 3) the generic lot that exhibited a wide difference in bioavailability compared to the reference product. The peak plasma levels were significantly higher for test product than reference.

Figure 3: Mean concentration-time profiles of 5 mg glyburide following administration of test and reference tablet to 26 healthy subjects, error bars represent ± SD.

The 90% confidence intervals of T/R ratio of the C\textsubscript{max}, AUC\textsubscript{0-24}, AUC\textsubscript{0-\infty}, were outside the bioequivalence acceptable range of 80-125% and therefore two products were concluded bioinequivalent (Table 4). The pharmacokinetic parameters and ANOVA was calculated using WinNonlin Professional program (Pharsight Corporation, 299 California Avenue, Palo Alto, CA 94306). The mean value of C\textsubscript{max}, AUC\textsubscript{0-24}, AUC\textsubscript{0-\infty}, T\textsubscript{max}, K\textsubscript{o}, T\textsubscript{1/2} for test product was 175.9 ng/mL, 782.6 ng.h/mL, 870.4 ng.h/mL, 3.25 h, 0.4387 h\textsuperscript{-1}, 2.49 h and that for reference was 107.9 ng/mL, 543.3 ng.h/mL, 622.3 ng.h/mL, 0.4098 h\textsuperscript{-1}, 2.93 h, respectively.

CONCLUSIONS

This simple, rapid and validated high performance liquid chromatographic method with single step sample preparation is permitting to process at least 60 samples a day. The method is successfully applied to about 700 samples collected for a bioequivalence study. The stability results indicated that glyburide and IS are quite stable in plasma and in the solutions therefore no special precautions are needed during sample treatment and storage. Because of high sensitivity and 0.5 ml sample requirement, this method can be used in pharmacokinetics, bioavailability and bioequivalence studies.

ACKNOWLEDGMENTS

We wish to thank Dr. Zaid Salhab of Prince Fahd Bin Sultan Hospital for his guidance in instituting norms of Good Clinical Practice, particularly, the ethical issues and documentation involved in conducting bioequivalence studies.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Least Squares Mean (LSM)</th>
<th>LSM ratio</th>
<th>90%CI</th>
<th>Prob. (&lt;80%)</th>
<th>Prob. (&gt;125%)</th>
<th>Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ln (C\textsubscript{max})</td>
<td>5.0667</td>
<td>4.6179</td>
<td>156.6</td>
<td>134.88-181.91</td>
<td>0.0000</td>
<td>0.9972</td>
</tr>
<tr>
<td>Ln (AUC\textsubscript{0-24})</td>
<td>6.5003</td>
<td>6.2066</td>
<td>134.1</td>
<td>114.71-156.87</td>
<td>0.0000</td>
<td>0.8825</td>
</tr>
<tr>
<td>Ln (AUC\textsubscript{0-\infty})</td>
<td>6.6267</td>
<td>6.3559</td>
<td>131.1</td>
<td>114.91-149.52</td>
<td>0.0000</td>
<td>0.8694</td>
</tr>
</tbody>
</table>
REFERENCES