Development and Validation of RP-HPLC Method for Analysis of Glipizide in Guinea Pig Plasma and its Application to Pharmacokinetic Study

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Abstract: A simple, stability-indicating reversed-phase high-performance liquid chromatographic (RP-HPLC) method has been developed for determination of Glipizide in guinea pig plasma. The method employed precipitation and selective extraction of Glipizide by ethyl acetate from the plasma followed by reversed-phase liquid chromatographic analysis with ultraviolet (UV) detection at 275 nm. Samples containing Glipizide were chromatographed on a PC-Micra NPS RP18 column with Phosphate Buffer (pH 3.5), acetonitrile, and THF in 80:15:5 as mobile phase at a flow rate of 0.4 mL min⁻¹. The retention time under these chromatographic conditions was found to be 2.01 minutes with run time 4.00 minute. Ethyl acetate was found to be good extracting and produced a satisfactory chromatogram, with extraction efficiency of 64% ± 4%. Bench-top stability study at room temperature for 48 h demonstrated that the Glipizide was with accuracy of 98.65 ± 3.2%. The inter-day and intraday coefficients of variation were less than 2%. The LOD and LOQ were found to be 6.45 ng/ml and 18.6 ng/ml respectively. The developed RP-HPLC method was found to be selective, simple, sensitive, accurate and linear for the analysis of Glipizide in guinea pig plasma. The retention time and in-turn run time was very short, hence required less mobile phase for the method, making it more economical and rapid. The developed method was used for the pharmacokinetic study of Glipizide in guinea pigs.

Key words: Glipizide, Guinea pig.

Introduction
Glipizide (1-Cyclohexyl-3-[[p-[2-(5- methylpyrazine carboxamido) ethyl] phenyl] sulfonyl] urea) is second generation sulfonylurea derivative synthesized in 1971 and used in the treatment of non-insulin dependent diabetes mellitus¹. This compound appears to be the most potent amongst other sulfonylurea derivatives ². The role of analytical method is very crucial in the success of in-vivo studies of any formulation. The sensitivity of the analytical method should be very high especially for in-vivo studies because the amount of the drug present in the plasma is usually very less and there are many other substances present in plasma, which may interfere with the analysis.

Literature revealed some of the methods used for the analysis/detection of Glipizide in plasma. Hartvig, reported the electron-capture gas chromatography of plasma sulfonylureas after extractive methylation.³ The Glipizide derivative was determined by electron-capture gas chromatography up to about 20ng/ml in a plasma sample. Emilsson had reported reverse phase HPLC method for analysis of Glipizide in human plasma and urine with the retention time of 9.67min.⁴ Shenfield et al described a technique for detecting most of the common sulfonylurea drugs in plasma. The method was semi-quantitative but the sensitivity of the assay was sufficient to detect and identify concentrations of the drugs.⁵ Nunez et al evaluated...
Micellar electrokinetic chromatography (MEKC) as a potential analytical method for the separation and detection of a series of sulfonylurea drugs used in the treatment of hyperglycemia with the detection limit of 50 ng/mL range. Magni et al described a method for identification of sulfonylureas in serum by electrospray mass spectrometry with LOD and LOQ about 2 and 10 ng/ml respectively. Dhawan and Singla, had developed RP-HPLC method for the determination of Glipizide in in-vitro as well as in-vivo samples, having retention time of about 9 min. Ho et al reported the method using similar technique, which can detect and confirm the 10 anti-diabetic drugs at 10 ng/ml each in equine plasma and equine urine. AbuRuza et al developed the solid phase extraction and HPLC methods for the simultaneous determination of metformin, Glipizide, gliclazide, glibenclamide and gliperide in plasma. Ding et al. developed the method for simultaneous determination of metformin and Glipizide in human plasma by liquid chromatography-tandem mass spectrometry.

The HPLC methods reported in the literature were having the retention time (RT) in the range of 7-12 minutes and the method run time more than 10 minutes. This high RT and run time of method make the analysis time consuming and costly. Moreover no method is reported for extraction and analysis of Glipizide in guinea pig plasma. In the present study was design to develop and validate a new RP-HPLC method for the analysis of Glipizide in guinea pig plasma. The developed method was used for the pharmacokinetic study of developed transdermal formulations of Glipizide in guinea pig.

**Experimental**

**Materials:** Pure Glipizide was obtained as gift sample from USV Limited, Mumbai, acetonitrile, methanol potassium dihydrogen orthophosphate (HPLC grade) were purchased from Merck Limited. Water used for chromatography was purified by a reverse osmosis water treatment plant (S.G.). All solvents and sample solutions were filtered through 0.22µm nylon filter (Millipore) before analysis. Extraction of Glipizide from plasma:

Glipizide present in plasma was extracted by liquid-liquid extraction method and reconstituted in a suitable medium for the analysis. To the 200 µl spiked plasma, 50 µl of 0.1 N HCl was added to precipitate out Glipizide and the sample was vortexed for 1 minute. Then Glipizide was extracted by vortexing with 1 ml of ethyl acetate and subjected to centrifugation at 5000 rpm for 5 minutes, to separate the ethyl acetate layer. Then 0.8ml of ethyl acetate layer was removed gently with micro-pipette and transformed to the HPLC vials. The eluent was then evaporated to dryness under nitrogen stream in a desiccators and residue was reconstituted in 100 µl mixture of phosphate buffer (4.5 pH) and methanol (4:6). The solutions after reconstitution were filtered through 0.22µm nylon filter (Millipore) before analysis. Extraction efficiency was determined by comparing the peak area of known amounts of Glipizide (un-extracted in reconstitution medium) with the peak area of samples containing the same amounts of Glipizide in plasma after extraction (Rajendran et al, 2007).

**Preparation of standard solution**

A stock solution was prepared by dissolving accurately weighed quantity of Glipizide in methanol to yield a final concentration of 1 mg /ml. The solution was sonicated for 5 minutes to ensure complete dissolution and allowed to equilibrate to room temperature after which it was suitably diluted with methanol. From the stock solution, working standards having concentration 100, 300 and 500 µg/ml of Glipizide were prepared by suitable dilution with methanol.

**Preparation and optimization of Mobile phase:**

Different ratios of phosphate buffer (PB), methanol, THF and acetonitrile were tried as mobile phase to get optimal retention time and other peak parameters. The composition and pH of mobile phase was optimized by several preliminary experimental trials to achieve good peak symmetry and short retention time.

**Construction of calibration curve**

2 µl of each working standard solution was spiked into 200 µl of guinea pig plasma to give 1, 3 and 5 µg/ml in plasma. The extraction and reconstitution of Glipizide was done as per the method described earlier. 10 µl of each of the above reconstituted samples (1, 3 and 5 µg/ml in plasma) were injected and peak area was obtained. A calibration curve was constructed by plotting peak areas versus concentrations.

**System suitability tests**

Through out the study, the suitability of the chromatographic system was monitored by calculating...
the trailing/ asymmetry factor, theoretical plates and relative standard deviation.

**Bench top stability studies**
To observe the possibility of interaction of drug with the other components bench top stability studies were carried out in triplicate. Glipizide at the concentration of 100ng/ml was prepared in guinea pig plasma and stored at room temperature for 48 h and then extracted and analyzed as above.

**Limits of Detection and Quantification**
The limit of detection (LOD) is the smallest amount of a sample that can be differentiated from background noise but not quantified. LOD was determined as the amount for which the signal-to-noise ratio (S/N) was 3:1 by comparing results from samples of known concentration with results from blank samples. The limit of quantification (LOQ) was defined as the lowest concentration of analyte that could be determined with acceptable precision and accuracy and was established by determining the concentrations of four spiked calibration standards.

**Inter-day and intraday accuracy and precision**
Intraday and inter-day accuracy was checked by analyzing the solutions at day 1, 3, and 5 which were stored at -20\(^\circ\)C. The percent error values were calculated by following equation.

\[
\text{Percent error} = \frac{\text{Observed concentration} \times 100}{\text{Added concentration}}
\]

Application to Pharmacokinetic Study
The protocol for the *in-vivo* animal experiments was reviewed and approved by Institutional Animal Ethical Committee (IAEC) (approval letter No: GCPA/ IAEC/2006/343). Guinea pigs of both sex, weighing around 0.8-1.0 kg were orally administered with Glipizide dispersed in distilled water (5mg/kg) with the help of 0.5 %w/v sodium carboxymethyl cellulose (CMC). Blood samples were withdrawn before the treatment and at 0.5,1,2,3,6,12,24 h after the treatment from the orbital sinus using disposable syringe (Rao et al, 2003). The blood samples were transformed into a centrifuge tube containing 3.2% w/v sodium citrate buffers solution as an anticoagulant. The Glipizide was extracted from the blood samples with the procedure described above, reconstituted and analyzed by developed HPLC method. Plasma concentration time profile curves and other pharmacokinetic parameters were calculated by using the mathematical software developed in MATHCAD.

Results and Discussion
Representative chromatograms obtained from a spiked serum sample from the pharmacokinetic study are shown in Fig-1. The results of optimization studies showed that the mixture of PB (pH 3.5), acetonitrile, and THF in 80:15:5 proportions give satisfactory results. The pH of mobile phase was adjusted to 3.5 with o-phosphoric acid. The optimized flow rate was found to be 0.4ml/min. The retention time under these chromatographic conditions was found to be 2.017 minutes with total run time of 4.00 minute. No endogenous interfering peaks were observed in blank serum of guinea pig confirming the specificity of the method.

Ethyl acetate was found to be a good extracting solvent and produced a satisfactory chromatogram (Fig-1). Glipizide is a weakly acidic drug, addition of 0.1 N HCl causes precipitation of Glipizide from the plasma. The extraction efficiency was 64% ± 4%.with 50 µl of HCl per 200 µl of plasma.

A three point calibration curve was constructed and found to be linear (Fig-2). The slope was calculated using the plot of drug concentration versus average peak area of the chromatogram. The correlation of coefficient ($r^2$) was found to be 0.9988.

System suitability parameters such as trailing/ asymmetry factor and relative standard deviation (RSD) were found to be well within acceptable limits (Table 1).

The inter-day and intraday coefficients of variation were less than 2% (Table-2), over the range of concentrations from 200-1000 ng/ml and accuracy was in the range of 98-99%.

The LOD and LOQ were found to be 6.45 ng/ml and 18.6 ng/ml respectively.

The method was used for analysis of Glipizide in guinea pig serum samples. After Single oral dose of 5mg/kg, Glipizide was extracted from the blood samples with the procedure described above, reconstituted and analyzed by developed HPLC method. Plasma concentration time profile curve (Fig-
2) and other pharmacokinetic parameters (Table-3) were calculated by using the mathematical software developed in MATHCAD.

**Conclusion**

A simple, sensitive, economic, and reliable RP-HPLC method has been developed and validated for determination of Glipizide in guinea pig serum. The method entails sample preparation by precipitation with acid, extraction with ethyl acetate, then chromatographic separation with UV detection. No interfering peaks were observed at the elution times of Glipizide. The method is accurate, reproducible, and specific. The retention time and in-turn run time was very short, hence required less mobile phase for the method, making it more economical and rapid. The method may be applicable for pharmacokinetic studies of Glipizide in guinea pig as a part of in-vivo studies of the developed formulations.

### Table 1: System suitability parameters of the method.

<table>
<thead>
<tr>
<th>Parameters (recommended values)</th>
<th>Observed values</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trailing/asymmetry factor (&lt;2.00)</td>
<td>1.4 ± 0.003</td>
<td>Complies</td>
</tr>
<tr>
<td>Theoretical plates (&gt; 2000)</td>
<td>638 ± 4</td>
<td>*</td>
</tr>
<tr>
<td>RSD (USP: RSD&lt; 2.0)</td>
<td>0.48 %</td>
<td>Complies</td>
</tr>
</tbody>
</table>

Values are mean ± S.D., n=3, * = may be due to the short length of column

### Table -2: Intraday and Inter-day precision and accuracy data.

<table>
<thead>
<tr>
<th>Added Conc. (ng/ml)</th>
<th>Intra-day Obs. conc. (ng/ml)</th>
<th>CV %</th>
<th>Accuracy (%)</th>
<th>Inter-day Obs. conc. (ng/ml)</th>
<th>CV (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>196.92</td>
<td>0.44</td>
<td>98.45</td>
<td>196.55</td>
<td>0.39</td>
<td>98.27</td>
</tr>
<tr>
<td>500</td>
<td>494.55</td>
<td>0.11</td>
<td>98.90</td>
<td>494.47</td>
<td>0.17</td>
<td>98.89</td>
</tr>
<tr>
<td>1000</td>
<td>992.42</td>
<td>0.23</td>
<td>98.64</td>
<td>990.60</td>
<td>0.29</td>
<td>98.12</td>
</tr>
</tbody>
</table>

CV=coefficient of variance; Obs. Conc. = Observed concentration.

### Table-3 Pharmacokinetic Parameters of Glipizide in guinea pig plasma after oral administration.

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>Value (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC₀→₂₄</td>
<td>70.962 ± 5.2</td>
</tr>
<tr>
<td>AUC₀→∞</td>
<td>76.127 ± 4.1</td>
</tr>
<tr>
<td>Kₑ</td>
<td>0.119 ± 0.018</td>
</tr>
<tr>
<td>t½</td>
<td>5.82 ± 0.32</td>
</tr>
<tr>
<td>Cₘₕₐₓ</td>
<td>11.2921 ± 0.58</td>
</tr>
<tr>
<td>Tₘₚₓ</td>
<td>0.00 ± 2.00</td>
</tr>
<tr>
<td>AUMC₀→₂₄</td>
<td>507.206 ± 26.2</td>
</tr>
<tr>
<td>AUMC₀→∞</td>
<td>631.773 ± 42.1</td>
</tr>
<tr>
<td>MRT</td>
<td>8.299 ± 0.76</td>
</tr>
</tbody>
</table>

Values are mean ± S.D., n= 6,
Fig- 1: Chromatogram of Glipizide extracted from plasma.

Fig-2: Standard calibration curve of Glipizide in guinea pig plasma. (Avg. of three readings)

Fig3: Plasma concentration time profile curve of Glipizide in guinea pig plasma.
References


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