### List of materials

<table>
<thead>
<tr>
<th>Drugs/excipients/solvents</th>
<th>Suppliers/manufacturers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gliclazide</td>
<td>Lupin Research Park, Pune</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>US Vitamins, Mumbai</td>
</tr>
<tr>
<td>Methanol (HPLC grade)</td>
<td>Finar Chemicals Ltd., Ahmedabad</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>Qualigens, Mumbai</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>Merck Specialities Pvt. Ltd., Mumbai</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>Nice Chemicals Pvt. Ltd., Cochin</td>
</tr>
<tr>
<td>Di sodium hydrogen phosphate</td>
<td>HiMedia Laboratories Pvt. Ltd., Mumbai</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>Sd. Fine Pvt. Ltd., Mumbai</td>
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<tr>
<td>Sodium acetate trihydrate</td>
<td>Suvidhinath Laboratories, Baroda</td>
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<tr>
<td>Ammonium acetate</td>
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<td>Polyethylene glycol 400</td>
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<td>Polyethylene glycol 600</td>
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<td>Tween 80</td>
<td>Loba Chemie Pvt. Ltd., Mumbai</td>
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<td>Tween 40</td>
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<td>Tween 20</td>
<td>Merck Specialities Pvt. Ltd., Mumbai</td>
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<tr>
<td>Span 80</td>
<td>Suvidhinath Laboratories, Baroda</td>
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<td>Boric acid</td>
<td>Sd. Fine Pvt. Ltd., Mumbai</td>
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<tr>
<td>Potassium chloride</td>
<td>Merck Specialities Pvt. Ltd., Mumbai</td>
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<td>Labrafil M2125C8</td>
<td>Ranbaxy Laboratories, Gurgaon</td>
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<td>Labrasol</td>
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<td>Lauroglycol 90</td>
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<td>Lauroglycol FCC</td>
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<td>Olive oil</td>
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<td>Soyabean oil</td>
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<td>Palm oil</td>
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<td>Peanut oil</td>
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<td>Corn oil</td>
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<tr>
<td>Cottonseed oil</td>
<td>Genuine Chemicals Co., Mumbai</td>
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<tr>
<td>Sesame oil</td>
<td>National Chemicals, Vadodara</td>
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</table>

All other excipients used were of analytical grade.
**List of instruments/equipments**

<table>
<thead>
<tr>
<th>Instruments/Equipments</th>
<th>Supplier/Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-Visible spectrophotometer</td>
<td>UV-1601PC, Shimadzu, Japan</td>
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<tr>
<td>HPLC</td>
<td>LC-2010CHT, Shimadzu, Japan</td>
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<td>Rotospin</td>
<td>Tarsons, Kolkata</td>
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<td>Water bath shaker</td>
<td>Remi Equipments Ltd., Bangalore</td>
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<tr>
<td>Cold centrifuge</td>
<td>Remi Equipments Ltd., Bangalore</td>
</tr>
<tr>
<td>USP dissolution apparatus</td>
<td>Electrolab, Mumbai</td>
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<tr>
<td>Spinwin</td>
<td>Tarsons, Kolkata</td>
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<tr>
<td>pH meter</td>
<td>Systronics µ pH system 361, Mumbai</td>
</tr>
<tr>
<td>Zetasizer Nano ZS</td>
<td>Malvern Instruments, UK</td>
</tr>
<tr>
<td>Viscometer</td>
<td>LV Brookfield, USA</td>
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<tr>
<td>Infrared spectrophotometer</td>
<td>FTIR 8300, Shimadzu, Japan</td>
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<tr>
<td>Microscope</td>
<td>SDMTECH, Andhra Pradesh</td>
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<tr>
<td>Halogen moisture analyser</td>
<td>Mettler Toledo (HB 43), USA</td>
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<tr>
<td>Digital electronic balance</td>
<td>Eaasae-Teraoka Ltd., Bangalore</td>
</tr>
<tr>
<td>Ultrasonic cleaning bath</td>
<td>Spectrolab, Delhi</td>
</tr>
<tr>
<td>Stability chamber</td>
<td>Thermolab, Mumbai</td>
</tr>
<tr>
<td>Transmission electron microscope</td>
<td>Philips Technai 12, The Netherlands</td>
</tr>
</tbody>
</table>
3.1. Analytical and bioanalytical method development for gliclazide

3.1.1. Identification of gliclazide

Analysis by UV spectrophotometer

In the present study estimation of gliclazide was carried out by uv-visible spectrophotometric method (UV-1601PC, Shimadzu Corporation, Japan). The drug solution was scanned in between the wavelength of 400 - 200 nm.

Solubility

Solubility of gliclazide was determined in water, alcohol, acetone and dichloromethane as per pharmacopoeial requirements.

Infrared spectroscopy

Gliclazide was confirmed by IR spectroscopy - fourier transform infrared (FTIR) spectrum using Shimadzu 8300 spectrometer and Hyper IR software from Shimadzu, Japan. The drug sample was dispersed in the KBr (200-400 mg) using a mortar, triturating the material into fine powder, and compressing the powder bed into the holder using a compression gauge with 140 mps pressure. The pellet was placed in the light path and the spectrum was recorded. The characteristic peaks of the functional groups were interpreted and compared with IR spectrum as given in pharmacopoeia.

Loss on drying

It was tested using Halogen Moisture Analyzer (Mettler Toledo, USA). 1 g of gliclazide was kept on pan for 10 min at 105 °C. Percentage LOD was reported.

3.1.2. Development and validation of reverse phase HPLC method for estimation of gliclazide

Gliclazide is a sulfonyl urea derivative having a molecular weight of 323.41 g/mol with a pKa of 5.8. It is a second-generation sulphonylurea drug, but unlike other drugs in this family, gliclazide contains an azabicyclo-octyl ring.
3.1.2.a. Method development

**Initial separation conditions**

Acetonitrile was selected as organic solvent to elute gliclazide from the stationary phase because of its favorable UV transmittance, low viscosity and low backpressure. Diluted concentration of 10 µg/ml gliclazide was prepared from the primary stock solution using methanol as a diluent.

- **Stationary phase**: Grace Vydac C<sub>18</sub> column (250 x 4.6 mm, 5µ)
- **Mobile phase**: Acetonitrile:20 mM ammonium acetate buffer, pH 4.5(50:50)
- **Run time**: Isocratic run for 20 min
- **Detection wavelength**: 230 nm
- **Flow rate**: 1 ml/min
- **Injection volume**: 20 µl
- **Temperature**: Ambient (around 25 °C)
- **Auto sampler temperature**: 4±2 °C

The standard solution of gliclazide 10 µg/ml was prepared, injected into HPLC system and run for 30 min.

**Effect of pH**

The mobile phase pH was optimized by using different pH conditions (pH 3.0 to pH 7.0) at a flow rate of 1 ml/min and Grace Vydac C<sub>18</sub> column used as the stationary phase.

**Effect of ratio of mobile phase**

Acetonitrile and ammonium acetate buffer (pH 4.5) were studied at 50:50, 60:40 and 70:30 (% v/v) for the proper selection of the mobile phase.

**Effect of ionic strength**

The Ammonium acetate buffer (pH 4.5) was prepared in different strength such as 20, 30 and 40 mM and chromatograms were recorded.

**Effect of flow rate**

The flow rate of 0.9, 1.0 and 1.1 ml/min were used and chromatograms were recorded.
3.1.2.b. Method validation (ICH Q2 R1 guidelines)

Linearity

Solutions with concentration of 0.5, 1, 2, 5, 10, 25, and 50 µg/ml of gliclazide was prepared in mobile phase and a volume of 20 µl of the solution was injected and chromatograms were recorded under the optimized chromatographic conditions. Peak area of the gliclazide was plotted against the concentration to get the regression equation and coefficient determination.

Accuracy

The known amount of standard drug was spiked (100, 120, 150%) in triplicate to the preanalyzed samples and the recovery of the drug was calculated at three concentrations such as 10, 12, 15 µg/ml.

Robustness

Evaluation of robustness leads to generation of a series of system suitability parameters which ensure that the analytical procedure is maintained whenever used.

Precision

Repeatability

A solution containing 10 µg/ml of gliclazide was analyzed at different time intervals and the percentage relative standard deviation was calculated.

Intermediate precision

It is generally expressed as the percent relative standard deviation for a statistically significant number of samples. It was carried out in between days and by different analysts.

Limit of detection (LOD) and Limit of quantitation (LOQ)

LOQ and LOD were calculated based on the standard deviation of slope and blank response from the calibration curve as per ICH guidelines.

\[
\text{LOD} = 3.3 \times \text{SD/S} \\
\text{LOQ} = 10 \times \text{SD/S}
\]

SD: Standard deviation of blank response; S: Slope of the calibration curve

3.1.3. Development and validation of bioanalytical method for estimation of gliclazide
3.1.3.a. Method development

Chromatographic conditions

Stationary phase : Grace Vydac C18 column (250 × 4.6 mm, 5μ)
Mobile phase : Acetonitrile: 20 mM ammonium acetate buffer, pH 4.5 (55:45)
Run time : Isocratic run for 20 min
Detection wavelength : 230 nm
Flow rate : 1 ml/min
Injection volume : 20 μl
Temperature : Ambient (around 25 °C)
Auto Sampler temperature : 4±2 °C

Collection of rat plasma

Blood samples from retro-orbital vein of male wistar rats (untreated with any drug) were collected using heparin capillary and was transferred to vacutainers containing EDTA. Vacutainers were centrifuged at 10000 rpm at 4 °C for 5 min. The supernatant was pipetted and stored at -70 °C.

Selection of internal standard

Different standard drugs like glibenclamide, glimepiride, glipizide were chromatographed in the same chromatographic condition. Glimepiride was well resolved from gliclazide with sharp symmetrical peak shape. Hence, in the present study, glimepiride was selected as an internal standard.

Optimization of extraction procedure of gliclazide from the rat plasma

The protein precipitation method was tried using 10% perchloric acid, 20% trichloroacetic acid, acetonitrile, methanol and its combination for the extraction of gliclazide from the rat plasma.

Extraction of gliclazide from rat plasma
95 µl of blank rat plasma was taken in the 1.5 ml microcentrifuge tube and 5 µl of glimepiride working stock solution (50 µg/ml) was added. Then the mixture was vortexed for 1 min. 250 µl of methanol was added, and vortexed for 10 minutes and then centrifuged at 10000 rpm for 10 min. 50 µl of clear supernatant liquid was separated and injected into HPLC for analysis.

3.1.3.b. Bioanalytical method validation (As per USFDA guidelines)

**Preparation of calibration curve and quality control samples**

A volume of 5 µl of working stock solutions were added to 95 µl of blank rat plasma and 10 µl IS solution (50 µg/ml) to get gliclazide concentrations 0.2, 0.3, 0.5, 1, 2, 5, 10, 25 and 50 µg/ml. Similarly 5 µl of controls were spiked with 95 µl of blank (drug free) rat plasma to get a gliclazide concentrations 0.2 µg/ml (LLOQ), 0.6 µg/ml (low), 20 µg/ml (medium), 40 µg/ml (high). These plasma samples were processed.

Each validation run consisted of a double control, system suitability sample, blank samples (a plasma sample processed with IS), calibration curve consisting of nine non-zero samples covering the total range (0.2–50 µg/ml) and QC samples at three concentrations (n = 6, at each concentration). Such validation runs were generated on four consecutive days. Calibration samples were analyzed from low to high at the beginning of each validation run and other samples were distributed randomly through the run.

**Selectivity**

Selectivity was established by injecting six samples at the LLOQ level and each of the six blank plasma samples were tested for interference by comparing the mean peak response obtained by injecting blank plasma samples to that of mean peak response of LLOQ (0.2 µg/ml).

**Recovery**

Recovery of gliclazide was evaluated by comparing the mean peak areas of three extracted low, medium and high quality control samples to mean peak areas of three neat reference solutions (unextracted ). Recovery of Glimepiride (IS) was evaluated by comparing the mean peak areas of extracted samples to mean peak areas of neat reference solutions (unextracted) of the same concentration.
Accuracy and precision

The intra-day precision and accuracy were tested by six replicates of each LLOQ, low, medium and high QC samples were prepared and injected. Accuracy was calculated based on the mean observed concentration as compared to the nominal concentration. The inter-day precision and accuracy were estimated from the assays of QC samples on four consecutive days.

Stability

In order to determine the stability of gliclazide in rat plasma, the samples were stored at four different stability conditions such as room temperature, freeze thaw stability, auto injector stability and long term stability which were examined by replicate analysis of the low and high plasma QC samples. Room temperature stability was carried out by keeping replicates of the low and high plasma quality control samples for approximately 24 h. Freeze–thaw stability of the samples was obtained over three freeze-thaw cycles, by thawing at room temperature for 2–3 h and refrozen for 12–24 h for each cycle. Auto sampler stability of gliclazide was tested by analysis of processed and reconstituted low and high plasma QC samples, which were stored in the auto sampler tray for 24 h. Long term stability of gliclazide in rat plasma was tested after storage at approximately −70 °C for 30 days. For each concentration and storage conditions, six replicates were analyzed in one set of batch. The results were compared to the data of freshly prepared and processed set of QC samples.

3.1.4 Results and discussion

3.1.4.1 Identification of gliclazide

UV Spectrum

A solution of 10 µg/ml gliclazide was scanned in the UV range of wavelength 400 - 200 nm. The λ_max was found to be 230 nm.

Solubility
Practically insoluble in water (0.13 mg/ml), soluble in methanol (40 mg/ml) and ethanol (35 mg/ml), sparingly soluble in acetone (5 mg/ml), freely soluble in dichloromethane (20 mg/ml). The solubility data complies with the pharmacopoeial specifications.

**IR Spectroscopy**

The presence of characteristic peaks associated with specific structural characteristics of the drug molecule was noted. The wave numbers for gliclazide with maximum peak intensities was found to be at 1701, 1346, 1161, 2866, 2943, 3111, 1697, 1595, 3271 cm⁻¹ respectively. The characteristic peaks revealed that the drug substance could be gliclazide. The IR spectrum of gliclazide was shown in Fig. 3.1.

![IR spectrum of gliclazide](image)

**Fig. 3.1.** IR spectrum of gliclazide.

**Loss on drying**

As per BP, LOD of gliclazide limit is NMT 0.25% when determined on 1.0 g by drying in an oven at 100 – 105 °C for 5 hr. LOD of the given sample of gliclazide was found to be 0.24% which complies with the BP specification

**3.1.4.2. Analytical method development and validation by reverse HPLC method**
**Effect of pH**

With the increase in pH of mobile phase there has been a decrease in the retention time of gliclazide and vice versa. So, Ammonium acetate buffer, pH of 4.5 was selected as the retention time was within 15 min.

**Effect of ratio of mobile phase**

The retention time of gliclazide was found to be 7.0, 6.4 and 3.1 mins respectively with different mobile phase ratio.

**Effect of ionic strength**

20 mM strength of Ammonium acetate buffer (pH 4.5) was selected as no change in the retention time was observed with the change in the ionic strength of the buffer.

**Effect of flow rate**

1 ml/min was selected was selected as good peaks were obtained with the all the different flow rates.

Based on the above optimization parameters, the following chromatographic condition was selected for the estimation of gliclazide by HPLC method consists of

- **Stationary phase**: Grace Vydac C18 column (250 × 4.6 mm, 5µ)
- **Mobile phase**: Acetonitrile: 20 mM ammonium acetate buffer, pH 4.5 (60:40)
- **Run time**: Isocratic run for 20 min
- **Detection wavelength**: 230 nm
- **Flow rate**: 1 ml/min
- **Injection volume**: 20 µl
- **Temperature**: Ambient (around 25 °C)
- **Autosampler Temperature**: 4±2 °C

With the above separation condition, the retention time for gliclazide was found to be 6.4 mins. The typical standard chromatogram of gliclazide was showed in the Fig. 3.2.
Linearity

The coefficient determination ($r^2$) for the present method was 0.9995 which indicated that the present method is linear and it is linear in the range from 10 – 70 µg/ml. Acceptance criteria for Linearity, ($r^2$) is >0.999. Calibration curve is shown in the Fig. 3.3.

Accuracy

The recoveries at three different concentrations (10, 12, 15 µg/ml) were found to be within the range of 98 to 102% as per ICH guidelines. Mean % recovery (Mean±SD) was found to be 99.62±1.43.

Robustness

The overall percentage relative standard deviation in the various parameters was found to be 0.92% and the acceptance limit was <2%. The result indicated that the method was robust.

Precision

The repeatability and intermediate precision of the proposed method was found to be 0.37% and 0.58% respectively which were within the acceptance criteria for the repeatability and intermediate precision (<1% and <2% RSD).

LOD and LOQ
The present method LOD and LOQ was found to be 0.1 and 0.5 µg /ml respectively. This indicates that the developed method is sensitive for the quantification of gliclazide. Summary of the analytical method validation parameters are reported in the Table 3.1.

**Table 3.1. Data of analytical method validation of gliclazide by HPLC.**

<table>
<thead>
<tr>
<th>Validation Parameters</th>
<th>Validation results</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity ($r^2$) (10-70 µg/ml)</td>
<td>0.9995</td>
<td>&gt; 0.999</td>
</tr>
<tr>
<td>Accuracy (% Mean±SD)</td>
<td>99.62±1.43</td>
<td>98–102</td>
</tr>
<tr>
<td>Robustness (% RSD)</td>
<td>0.92</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Repeatability precision (% RSD)</td>
<td>0.37</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Intermediate precision (% RSD)</td>
<td>0.58</td>
<td>&lt;2</td>
</tr>
<tr>
<td>LOD (µg/ml)</td>
<td>0.1</td>
<td>S/N ratio should be 3:1</td>
</tr>
<tr>
<td>LOQ (µg/ml)</td>
<td>0.5</td>
<td>S/N ratio should be 10:1</td>
</tr>
</tbody>
</table>

**Fig 3.3.** Calibration plot of gliclazide by HPLC method.
3.1.4.3. Bioanalytical method development and validation by reverse HPLC method

Protein precipitation method was selected for the extraction of gliclazide from the rat plasma. The developed method was able to extract 85% and also there were no interferences at the retention time of gliclazide and glimepiride.

The optimized chromatographic condition consists of the following:

- **Stationary phase**: Grace Vydac C\textsubscript{18} column (250 × 4.6 mm, 5\textmu m)
- **Mobile phase**: Acetonitrile: 20 mM ammonium acetate buffer, pH 4.5 (55:45)
- **Run time**: Isocratic run for 20 min
- **Detection wavelength**: 230 nm
- **Flow rate**: 1 ml/min
- **Injection volume**: 20 \textmu l
- **Temperature**: Ambient (around 25 °C)
- **Sample temperature**: 4±2 °C

**Selectivity**

Gliclazide and glimepiride (IS) were well separated from the plasma proteins under the optimized chromatographic conditions at retention time of 8.36 min and 12.41 min and the same were shown in the Fig. 3.4. The peaks were of good shape with good separation among each other and there were no interferences from the plasma matrix.

**Linearity**

Linear detector response for the peak-area ratios of the gliclazide to internal standard was observed in concentration range between 0.2–50 \textmu g/ml with a coefficient determination of 0.9992 and shown in Fig. 3.5.

**Lower Limit of Quantitation (Sensitivity)**

LLOQ 0.2 \textmu g/ml was selected based on the lowest level concentration that would be expected for analysis in the current study. Accuracy and precision was carried out and it was found to be within the acceptable limits.
Accuracy and precision of QC samples

Accuracy measures the percentage deviation of nominal concentration as compared to the observed concentration. The accuracy values for intra and interday at the LLOQ and at low, medium and high quality control samples of gliclazide in plasma were within acceptable limits (n=6). The results of the method validation study are presented in Table 3.2. All the results were within the acceptable limits i.e. 85 - 115% of nominal concentrations and the % CV for LLOQ and all QC were within 15%.

Extraction recovery

The proposed method (Mean percentage recovery ± SD) was found to be 76.11 ± 2.75%. The internal standard % recovery was found to be 78.31 ± 2.25%. The above values show that the extraction efficiency of the method was consistent.

Stability

The stability of the drug spiked at two QC levels (LQC, HQC), auto injector (24 h), bench top (8 h), freeze thaw (3 cycles) and long term (30 days) studies were evaluated. The results showed that the gliclazide was stable in plasma for about one month when stored in the frozen state (-70 °C).
Fig. 3.4. Standard chromatogram of gliclazide in rat plasma with internal standard (glimepiride).

Fig. 3.5. Calibration plot of gliclazide.

Table 3.2. Summary of bioanalytical method validation of gliclazide by HPLC.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Validation Values</th>
<th>Acceptance limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity</td>
<td>0.2-20 µg/ml, ( r^2 &gt; 0.999 )</td>
<td>&gt;0.98 with consistency</td>
</tr>
<tr>
<td>Intra batch accuracy (%)</td>
<td>92.41-100.17</td>
<td>85-115.0% except the LLOQ (80-120%)</td>
</tr>
<tr>
<td>Inter batch accuracy (%)</td>
<td>93.22-103.34</td>
<td>85-115.0% except the LLOQ (80-120%)</td>
</tr>
<tr>
<td>Intra Batch precision (% CV)</td>
<td>2.07-8.93</td>
<td>±15% dev from nominal conc. except at LLOQ ± 20% dev.</td>
</tr>
<tr>
<td>Inter Batch Precision (% CV)</td>
<td>6.23-10.25</td>
<td>±15% dev from nominal conc. except at LLOQ ± 20% dev.</td>
</tr>
<tr>
<td>Recovery analyte (mean±SD)</td>
<td>76.11±2.75</td>
<td>Consistent recovery</td>
</tr>
<tr>
<td>Recovery (Internal standard)</td>
<td>78.31±2.25</td>
<td></td>
</tr>
<tr>
<td>% stability by freeze thaw (-70 °C)</td>
<td>80.21-103.11</td>
<td>85 -115.0% except the LLOQ (80 - 120%)</td>
</tr>
<tr>
<td>% stability by auto injector stability (18 h) (4 °C)</td>
<td>93.19-105.28</td>
<td>85 -115.0% except the LLOQ (80-120%)</td>
</tr>
<tr>
<td>% stability by bench top stability (8 h)</td>
<td>94.36-105.49</td>
<td>85 -115.0% except the LLOQ (80 -120%)</td>
</tr>
<tr>
<td>% stability by long term</td>
<td>93.23-101.13</td>
<td>85 -115.0% except the LLOQ (80-120%)</td>
</tr>
</tbody>
</table>
The results of validation showed that the method was accurate, precise and selective and linear over the wide range of concentration. The method was very simple and was successfully applied to the determination of gliclazide in rat plasma.

3.2. Analytical and bioanalytical method development for glibenclamide

3.2.1. Identification of glibenclamide

Analysis by UV spectrophotometer

The drug solution of 10 µg/ml was scanned in the uv range and $\lambda_{\text{max}}$ was determined.

Solubility

The solubility of glibenclamide was estimated in water, methylene chloride, alcohol and methanol as per pharmacopoeial requirements.

Infrared spectroscopy

Glibenclamide sample was confirmed by IR spectroscopy using Shimadzu 8300 spectrometer and Hyper IR software from Shimadzu, Japan. The characteristic peaks were compared with IR spectrum as given in pharmacopoeia.

3.2.2. Development and validation of reverse phase HPLC method for estimation of glibenclamide

Glibenclamide is a sulfonyl urea derivative having a molecular weight of 494 g/mol with a pKa of 5.3. It is practically insoluble in water, sparingly soluble in methylene chloride, soluble in alcohol and in methanol.

3.2.2.a. Method development

Initial separation conditions

Acetonitrile was selected as organic phase to elute glibenclamide. 10 µg/ml glibenclamide was prepared from the primary stock solution using methanol as a diluent and injected in to HPLC system with the following chromatographic conditions.
Stationary phase: Grace Vydac C\textsubscript{18} column (250 × 4.6 mm, 5\textmu m)
Mobile phase: Acetonitrile: 20 mM ammonium acetate buffer, pH 4.5 (50:50)
Solvent ratio: Isocratic run for 30 min
Detection wavelength: 300 nm
Flow rate: 1 ml/min; Injection volume: 20 \mu l
Temperature: Ambient (around 25 °C); Auto sampler temperature: 4±2 °C

**Effect of pH, composition, ionic strength, flow rate of mobile phase**

The mobile phases with different pH conditions (pH 3.0 to pH 7.0) were used and retention time was noted. Various composition of mobile phase were studied at 50:50, 60:40 and 70:30 (% v/v) to optimize the composition of mobile phase. Different strength of ammonium acetate buffer (pH 4.5) such as 10, 20, 30 and 40 mM with varied flow rate such as 0.9, 1.0 and 1.1 ml/min were used and chromatograms were recorded.

**3.2.2.b. Method validation (ICH Q2 R1 guidelines)**

**Linearity**

The linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyte. 0.5, 1, 2, 5, 10, 25, and 50 \mu g/ml of glibenclamide was prepared in mobile phase and 20 \mu l of the solution was injected and chromatograms were recorded and regression equation was calculated.

**Accuracy**

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted as true value or reference value. Known amount of standard drug was spiked (100, 120, 150%) in triplicate to the preanalyzed samples and the recovery of the drug was calculated at 10, 12, 15 \mu g/ml.

**Robustness**

Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters. One consequence of evaluation of robustness should be that a series of system suitability parameters is established to ensure that the analytical procedure is maintained whenever used.
Precision

Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. It is also termed as intra-assay precision. 10 µg/ml of glibenclamide was analyzed at different time intervals. Percentage relative standard deviation was then calculated.

Intermediate precision

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is generally expressed as the percent relative standard deviation for a statistically significant number of samples. It was carried out in between days and by different analysts.

Limit of detection (LOD) and Limit of quantitation (LOQ)

LOD is ability of analytical method able to detect the lowest concentration of the analyte. LOQ is lowest concentration of the analyte which can be quantitatively analyzed with acceptable precision and accuracy. It was calculated based on the slope and blank response from the calibration curve as per ICH guidelines. LOD and LOQ were calculated based on the standard deviation of the response and slope.

\[
\text{LOD} = 3.3 \times \text{SD}/\text{S} \\
\text{LOQ} = 10 \times \text{SD}/\text{S}
\]

SD: Standard deviation of blank response; S: Slope of the calibration curve

3.2.3. Development and validation of bioanalytical method for estimation of glibenclamide

3.2.3.a. Method development

Chromatographic conditions

Stationary phase: Grace Vydac C\textsubscript{18} column (250 × 4.6 mm, 5µ)
Mobile phase: Acetonitrile: 20 mM ammonium acetate buffer, pH 4.5 (55:45)
Solvent ratio: Isocratic run for 30 min
Detection wavelength: 300 nm
Flow rate: 1 ml/min
Injection volume: 20 µl
Temperature: Ambient (around 25 °C)
Autosampler Temperature: 4±2 °C

Collection of rat plasma

The blood samples from retro-orbital vein of male wistar rats (untreated with any drug) was collected using heparin capillary and was transferred to vacutainers containing EDTA. Vacutainers were centrifuged at 10000 rpm at 4 °C for 5 min. The supernatant was pipetted and stored at -70 °C for further experimental use. Similarly diabetes induced rats blank blood was collected and plasma was separated and stored in the refrigerator.

Selection of internal standard

Selection of internal standard is mainly based on the chemical structure, solubility, polarity characteristics of the drug. Different standard drugs like gliclazide, glimepiride, glipizide were chromatographed in the same chromatographic condition. Glipizide was well resolved from glibenclamide with sharp symmetrical peak shape. Hence, in the present study, glipizide was selected as an internal standard.

Optimization of extraction procedure of glibenclamide from the rat plasma

The protein precipitation method was tried using 10% perchloric acid, 20% trichloroacetic acid, acetonitrile, methanol and its combination for the extraction of glibenclamide from the rat plasma.

Extraction of glibenclamide from rat plasma

95 µl of blank rat plasma was taken in the 1.5 ml microcentrifuge tube and 5 µl of glibenclamide with varying concentrations and 10 µl of IS (glipizide) working stock solution (50 µg/ml) was added. Then the mixture was vortexed for 1 min. 250 µl of methanol was
added, and vortexed for 10 minutes and then centrifuged at 10000 rpm for 10 min. 50 µl of clear supernatant liquid was separated and injected into HPLC for analysis.

3.2.3.b. Bioanalytical method validation (As per USFDA guidelines)

Preparation of calibration curve and quality control samples

A volume of 5 µl of working stock solutions were added to 95 µL of blank rat plasma and 10 µl IS solution (50 µg/ml) to get glibenclamide concentrations 0.2, 0.3, 0.5, 1, 2, 5, 10, 25 and 50 µg/ml. Similarly 5 µl of controls were spiked with 95 µl of blank (drug free) rat plasma to get a glibenclamide concentrations 0.2 µg/ml (LLOQ), 0.6 µg/ml (low), 20 µg/ml (medium), 40 µg/ml (high). These plasma samples were processed.

Each validation run consisted of a double control, system suitability sample, blank samples (a plasma sample processed with IS), calibration curve consisting of nine non-zero samples covering the total range (0.2–50 µg/ml) and QC samples at three concentrations (n = 6, at each concentration). Such validation runs were generated on four consecutive days. Calibration samples were analyzed from low to high at the beginning of each validation run and other samples were distributed randomly through the run.

Selectivity, recovery, accuracy, precision and stability

The method used was similar to as explained previously in the bioanalytical method validation of gliclazide in 3.1.3.2.

3.2.4. Results and discussion

3.2.4.1. Identification of glibenclamide

UV Spectrum

λ<sub>max</sub> was found to be 300 nm which complies with IP specification.

Solubility
The drug was found to be practically insoluble in water (0.05 mg/ml), sparingly soluble in methylene chloride (5 mg/ml), slightly soluble in alcohol (10 mg/ml) and in methanol (12 mg/ml). The solubility data complies with the pharmacopoeial specifications.

**IR Spectroscopy**

In order to confirm the functional group of the drug IR spectrum was taken. The wave numbers was found to be at 1714, 1342, 1155, 2852, 2929, 3115, 1525, 1618, 3365 cm\(^{-1}\) respectively. The characteristic peaks revealed that the drug substance could be glibenclamide. The IR spectrum of glibenclamide was shown in Fig. 3.6.

![Fig. 3.6. IR spectrum of glibenclamide.](image)

**3.2.4.2. Analytical method development and validation by reverse HPLC method**

**Effect of pH, composition, ionic strength, flow rate of mobile phase**

The retention time of glibenclamide was decreased with increase in pH of the mobile phase. This might be due to the unionization of the drug. Hence, Ammonium acetate buffer, pH of 4.5 was selected since elution was within 15 min with adequate system suitability. With different mobile phase ratio the retention time was found to be 8.4, 7.5 and 4.2 min.
respectively. On increasing the % of acetonitrile there was a decrease in the retention time which might be due to the higher elution strength of acetonitrile. The retention time of glibenclamide did not change much with the change in buffer strength. So, 20 mM strength of Ammonium acetate buffer (pH 4.5) was selected. Symmetrical peaks were obtained with the different flow rates. For the present study, 1 ml/min was selected. Based on the above optimization parameters, the following chromatographic condition was selected for the estimation of glibenclamide by HPLC method.

**Stationary phase**: Grace Vydac C<sub>18</sub> column (250 × 4.6 mm, 5µ)

**Mobile phase**: Acetonitrile: 20 mM ammonium acetate buffer, pH 4.5 (60:40)

**Solvent ratio**: Isocratic run for 30 min

**Detection wavelength**: 300 nm

**Flow rate**: 1 ml/min

**Injection volume**: 20 µl

**Temperature**: Ambient (around 25 °C)

**Autosampler Temperature**: 4±2 °C

With the above separation condition, the retention time for glibenclamide was found to be 7.5 mins. The typical standard chromatogram of glibenclamide was showed in the Fig. 3.7.

![Fig. 3.7. Standard chromatogram of glibenclamide.](image)

**Linearity**

Linearity is generally reported by the coefficient determination ($r^2$) and the acceptance criteria is ($r^2$) should be >0.999. The coefficient determination ($r^2$) was 0.9997 which
indicated that the present method is linear (range 10–70 µg/ml). Standard plot is shown in Fig. 3.8.

**Accuracy**

Recoveries at three different concentrations (10, 12, 15 µg/ml) were found to be within the range of 98 to 102% as per ICH guidelines. Mean % recovery (Mean±SD) was found to be 99.22±1.07.

**Robustness**

Overall percentage relative standard deviation was found to be 0.90% and the acceptance limit was <2% which indicated that the method was robust.

**Precision**

The repeatability and intermediate precision of the proposed method was found to be 0.56% and 0.72% respectively which were within the acceptance criteria indicating that the method was precise and reproducible one.

**LOD and LOQ**

LOD and LOQ were found to be 0.1 and 0.5 µg/ml which suggests that the developed method is sensitive for the quantification of glibenclamide. Summary of the analytical method validation parameters are reported in the Table 3.3.

**Table 3.3.** Data of analytical method validation of glibenclamide by HPLC.

<table>
<thead>
<tr>
<th>Validation Parameters</th>
<th>Validation results</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity (r²) (10-70 µg/ml)</td>
<td>0.9997</td>
<td>&gt; 0.999</td>
</tr>
<tr>
<td>Accuracy (% Mean±SD)</td>
<td>99.22±1.07</td>
<td>98–102</td>
</tr>
<tr>
<td>Robustness (% RSD)</td>
<td>0.90</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Repeatability precision (% RSD)</td>
<td>0.56</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Intermediate precision (% RSD)</td>
<td>0.72</td>
<td>&lt;2</td>
</tr>
<tr>
<td>LOD (µg/ml)</td>
<td>0.1</td>
<td>S/N ratio should be 3:1</td>
</tr>
<tr>
<td>LOQ (µg/ml)</td>
<td>0.5</td>
<td>S/N ratio should be 10:1</td>
</tr>
</tbody>
</table>
3.2.4.3. Bioanalytical method development and validation by reverse HPLC method

Protein precipitation method was selected for the extraction of glibenclamide from the rat plasma. The optimized chromatographic condition consists of the following:

- **Stationary phase**: Grace Vydac C18 column (250 × 4.6 mm, 5 μ)
- **Mobile phase**: Acetonitrile: 20 mM ammonium acetate buffer, pH 4.5 (55:45)
- **Run time**: Isocratic run for 30 min
- **Detection wavelength**: 300 nm
- **Flow rate**: 1 ml/min
- **Injection volume**: 20 μl
- **Temperature**: Ambient (around 25 °C)
- **Autosampler Temperature**: 4±2 °C

**Bioanalytical method validation of glibenclamide by HPLC**

**Selectivity**

Retention time for glibenclamide and glipizide (IS) were 10.04 and 4.3 min (Fig 3.9). The peaks were of good shape with good separation among each other without any interference.

**Linearity**

The coefficient determination was found to be 0.9993 in the concentration range of 0.2–20 μg/ml as shown in Fig 3.10.
Lower Limit of Quantitation (Sensitivity)

Based on lowest level concentration LLOQ 0.2 µg/ml was selected.

Accuracy and precision of QC samples

The accuracy values for intra and interday at the LLOQ and at low, medium and high quality control samples of glibenclamide in plasma were within acceptable limits (n=6). The results of the method validation study are presented in Table 3.4. All the results were within the acceptable limits.

Extraction recovery

Mean percentage recovery was found to be 75.18±3.25%. The internal standard % recovery was found to be 77.12±3.21%. The above values indicate that the extraction efficiency of the method was consistent.

Stability

Stability of the drug spiked at two QC levels (LQC, HQC), auto injector (24 h), bench top (8 h), freeze thaw (3 cycles) and long term (30 days) studies were evaluated. The results showed that the glibenclamide was stable in plasma for about one month when stored in the frozen state (- 70 ºC).

Fig.3.9. Typical standard chromatogram of glibenclamide in rat plasma with internal standard (glipizide).
The results suggest that method was very simple and was successfully applied for the determination of glibenclamide in rat plasma. Validation results showed that the method was accurate, precise and selective and linear over the wide range of concentration.