4. EXPERIMENTAL

This part of the thesis describes the sources and specifications of the active pharmaceutical ingredients and chemicals which were used in carrying out the present research work. This section is further divided into two parts: the first part includes the detailing of the process parameters used for the preparation of cocrystals of gliclazide, glipizide and repaglinide whereas the second part covers the various polymorphic forms of repaglinide. A detailed account of various analytical instruments and techniques adopted for the characterization and evaluation of cocrystals and polymorphs is also given.

4.1 Active Pharmaceutical Ingredients (APIs)

All the APIs were procured as gift samples from pharmaceutical manufacturing firms.

Sources

Gliclazide: Ind Swift Ltd., Panchkula, India & Consen Pharma Pvt. Ltd., Ludhiana, India

Glipizide: La Pharma Pvt. Ltd., Ludhiana, India

Repaglinide: Terrace Pharmaceutical Pvt. Ltd., Mohali, India

4.2 Chemicals and solvents

The sources and the grades of the chemicals and solvents, used in the present study are given in table 4.1.

Table 4.1: List of chemicals, their sources and grades

<table>
<thead>
<tr>
<th>Chemicals and solvents</th>
<th>Source</th>
<th>Grade</th>
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<tbody>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>Central Drug House Ltd., New Delhi</td>
<td>AR</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>S. D. Fine Chem. Ltd., Mumbai</td>
<td>AR</td>
</tr>
<tr>
<td>Isonicotinic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotinamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>Sigma-Aldrich-Merck Ltd., Mumbai</td>
<td>AR</td>
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<tr>
<td>Orotic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sebacic acid</td>
<td></td>
<td></td>
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<tr>
<td>Sorbic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptozotocin</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Trisodium citrate</td>
<td>Himedia Laboratories, Mumbai</td>
<td>AR</td>
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<tr>
<td>Monohydrate sodium citrate</td>
<td>Adenine</td>
<td>Adipic acid</td>
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</tbody>
</table>
### 4.3 Softwares

The following softwares were used in the present thesis work.

- ChemOffice 16.0, PerkinElmer
- ConQuest 1.7, CSD 5.36, 2014, Cambridge Crystallographic Data Centre (CCDC)
- Material Studio 7.0, BIOVIA
- Mercury 3.1, Cambridge Crystallographic Data Centre (CCDC)
- Encifer 1.5.1, Cambridge Crystallographic Data Centre (CCDC)
- GraphPad Prism 6.0, GraphPad Software, Inc.
- PK Solver 2.0

### 4.4 Buffers and their components

**Phosphate buffer pH-7.4**

50 mL of 0.2 M potassium dihydrogen phosphate (27.218 g in 1000 mL of distilled water) and 39.1 mL of 0.2 M sodium hydroxide (8 g in 1000 mL of distilled water) were mixed and volume was made up to 200 mL with distilled water.

**Citrate buffer pH 5**

0.7 g of citric acid and 1.86 g of trisodium citrate were mixed in 100 mL of distilled water. The final pH 5 was adjusted using monohydrate sodium citrate solution.

**Citrate buffer pH 4.4**

1 part of citric acid (1.5 g in 100 mL of distilled water) and 1 part of trisodium citrate (1.8 g in 1000 mL of distilled water) was mixed. The final pH 4.4 was adjusted using monohydrate sodium citrate solution.
4.5 Cocrystals of Gliclazide, Glipizide and Repaglinide

4.5.1 Designing of cocrystals

The foremost and important step in the designing of cocrystal is selection of a compatible coformer. For GL, GPZ and RPG, the coformers were selected by a supramolecular synthon approach (Sarma and Desiraju, 2002) which comprises the statistical analysis of the Cambridge Structural Database (CSD, version 5.36 November, 2014). The preliminary search for the propensity of functional groups that may generate supramolecular synthon was conducted using ConQuest (Bruno et al., 2002) software® (version 1.7). The lead for the designing of the new cocrystals of GL, GPZ and RPG was obtained through the statistics of matching of the functional groups present in APIs (GL: SO₂NH, CONH; GPZ: SO₂NH, CONH, pyrazine ring; RPG: piperidine ring, COOH, CONH) with other complementary functional groups of coformers (SO₂NH, COOH, CONH, NH₂, OH, pyrrole ring and pyridine ring). The data was obtained in the forms of “HITS” with constraints such as presence of 3D coordinates, R factor less than 0.05, no disordered structures, no errors, no ions, only organic compounds including powder structures. The scheme for the designing of cocrystals of GL, GPZ and RPG is given in figure 4.1.

![Scheme used for the selection of coformers](image)

**Figure 4.1:** Scheme used for the selection of coformers

4.5.2 Preparation of cocrystals

For the preparation of cocrystals of GL, GPZ and RPG, various GRAS coformers containing complementary functional groups were tried. However, the cocrystals were isolated only with the coformers having carboxylic acid functionality. GL formed cocrystals with succinic acid (SA), malic acid (MA), α-hydroxyacetic acid (HA) and sebacic acid (SB) whereas the
Experimental

cocrystals of GPZ were recognised with picolinic acid (PA), adipic acid (AA), isonicotinic acid (INA), fumaric acid (FA) and sorbic acid (SRA). RPG successfully cocrystallized with α-hydroxyacetic acid (HA), picolinic acid (PA), sebacic acid (SB), adipic acid (AA) and pyridoxine (PD). All the cocrystals were prepared using solvent drop grinding method which is a green and viable approach. The experiments for the re-crystallization of the prepared cocrystals from the various solvents were also carried out but the suitable crystals for single crystal X-ray diffraction analysis were not isolated.

**Gliclazide:** Four cocrystals of GL (Figure 4.2), namely GL-SA, GL-MA, GL-SB and GL-HA were prepared by grinding GL (323.41 mg, 1 mmol) separately with SA (118.09 mg, 1 mmol), MA (134.09 mg, 1 mmol), SB (202.25 mg, 1 mmol) and HA (76.05 mg, 1 mmol) respectively. The corresponding stoichiometric 1:1 mixtures were ground in pestle mortar for 1 h at room temperature with the drop wise addition of solvent. Ethanol (5 mL) was used in the preparation of GL-SA whereas acetone (10 mL) was used in the grinding of GL-MA, GL-SB and GL-HA. The dried final products were scratched out and stored in desiccators for further analysis.

![Scheme used for the preparation of cocrystals of GL](image)

**Figure 4.2: Scheme used for the preparation of cocrystals of GL**

**Glipizide:** Five cocrystals of GPZ (Figure 4.3), namely GPZ-PA, GPZ-AA, GPZ-INA, GPZ-FA and GPZ-SRA were prepared by grinding GPZ (445.54 mg, 1 mmol) separately with PA (123.11 mg, 1 mmol), AA (146.14 mg, 1 mmol), INA (123.11 mg, 1 mmol), FA (116.07 mg, 1 mmol) and SRA (112.13 mg, 1 mmol) respectively. The corresponding stoichiometric 1:1 mixtures were ground in pestle mortar for 2 h at room temperature with the drop wise addition of ethanol (10 mL). The obtained products were dried and stored in desiccators for further analysis.
Figure 4.3: Scheme used for the preparation of cocrystals of GPZ

**Repaglinide:** Five cocrystals of RPG (Figure 4.4), namely RPG-HA, RPG-PA, RPG-SB, RPG-AA and RPG-PD were prepared by grinding RPG (452.59 mg, 1 mmol) separately with HA (76.05 mg, 1 mmol), PA (123.11 mg, 1 mmol), SB (202.25 mg, 1 mmol), AA (146.14 mg, 1 mmol) and PD (169.18 mg, 1 mmol) respectively. The corresponding stoichiometric 1:1 mixtures were ground in pestle mortar for 1.5 h at room temperature with the drop wise addition of ethanol (10 mL). The obtained products were dried and stored in desiccators for further analysis.

Figure 4.4: Scheme used for the preparation of cocrystals of RPG
4.6 Polymorphs of Repaglinide

4.6.1 Preparation of polymorphs

Slow evaporation approach was used to prepare the different polymorphs of repaglinide. For this, saturated solution of Form I of RPG (commercial sample) was prepared in various solvents and mixture of solvents and subjected to evaporation at 25°C. The type of the obtained product and the time taken for crystallization is given in table 4.2.

Table 4.2: Crystallization products of RPG from different solvents

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Product</th>
<th>Time to obtain product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethylformamide</td>
<td>White crystals</td>
<td>7 days</td>
</tr>
<tr>
<td>Methyl acetate</td>
<td>White crystals</td>
<td>2 days</td>
</tr>
<tr>
<td>Propyl acetate</td>
<td>White crystals</td>
<td>3 days</td>
</tr>
<tr>
<td>n-butyl acetate</td>
<td>White crystals</td>
<td>2 days</td>
</tr>
<tr>
<td>Xylene</td>
<td>Crystalline solid</td>
<td>1 day</td>
</tr>
<tr>
<td>Acetylacetone</td>
<td>Crystalline solid</td>
<td>1 day</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>Crystalline solid</td>
<td>4 days</td>
</tr>
<tr>
<td>1,2-dichloroethane</td>
<td>Crystalline solid</td>
<td>2 days</td>
</tr>
</tbody>
</table>

The crystals obtained from dimethylformamide, methyl acetate, propyl acetate and n-butyl acetate were filtered and dried at room temperature whereas the crystalline solid obtained from xylene, acetylacetone, dichloromethane and 1,2-dichloroethane was dried at a temperature of 40°C. After drying, the products were characterized using various analytical techniques.

4.6.2 Crystal Structure Prediction of polymorphs of Repaglinide

4.6.2.1 Conformational searches

RPG molecule was sketched and optimized geometrically by smart algorithm in Forcite module using COMPASS forcefield. Later the conformational sampling of RPG was executed by conformer module of BIOVIA material studio. The systematic grid conformational search with geometry optimization and restraints method was done by varying one out of 11 torsion angles. The restraint force constant was kept at 1000 kcal/mol/rad^2. The torsions were varied from -180° to 180°, in 120 steps with 1.3° of interval. The search of geometrically optimized conformer with low energy was performed by atom based summation method using pcff forcefield. The perturbation in the energy of
conformers with the change in torsion angles was studied. The lowest energy conformers were re-optimized for energy by Dmol\textsuperscript{3} using gradient-corrected generalized gradient approximations (GGA) and PBE (Perdew-Burke-Ernzerhof) functional in density functional theory (DFT) calculations with double numeric plus polarization (DNP) basis set. The electrostatic potential (ESP) charge was generated on the re-optimized conformer

4.6.2.2 Global search
The identified stable conformers in the conformational search were subjected for search of global minima. The polymorph predictor module of BIOVIA material studio was used to estimate the crystal packing of different chosen conformers in most common space groups (P2\textsubscript{1}/c, P\textsubscript{1}, P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1}, P\textsubscript{2}1, C2/c, Pbcn, Pna\textsubscript{2}1, Pbcn, Cc, and C2). The prediction was performed by Ewald summation for electrostatic interactions and by atom based summation for the van der Waals forces using COMPASS forcefields. The polymorph prediction job serves the energy optimized potential crystal packing of various conformers in different space groups. The clustering was performed after both packing search and energy optimization step, to remove the duplicate entries. The crystal energy landscape (energy vs density graph) for all the potential polymorphs was plotted to gain an insight into the paradigm of RPG polymorphs.

4.7. Characterization of prepared cocrystals and polymorphs
4.7.1 Differential Scanning Calorimetry (DSC)
DSC, Q20 (TA-Instruments Waters, USA), equipped with a refrigerated cooling system (installed in University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh) was used for obtaining DSC scans. The DSC was calibrated with pure indium (mpt: 156.6 °C and ΔH: 25.45 Jg\textsuperscript{-1}). The samples (2-5 mg) were sealed in an aluminium pan and the empty aluminium pan was treated as reference pan. An inert atmosphere was created in the sample cell by passing the dry nitrogen with a flow rate of 50 mL/min. The samples were heated at the ramp rate of 10 °C/min in the temperature range of 25-250 °C for recording the thermal changes. The results were integrated by TA Q series Advantage software (Universal analysis 2000).

4.7.2 Powder X-ray Diffraction (PXRD)
X’Pert-Pro Diffractometer (PANalytical, Netherlands), installed in Sophisticated Analytical Instrumentation Facility (SAIF), Panjab University, Chandigarh was used for recording the PXRD pattern of the samples. Cu (anode tube) Kα radiation of wavelength 1.54060 Å was used for tracing the crystallinity pattern. The tube voltage and current were set at 40 kV and
40 mA respectively. The diffractograms were recorded with fixed divergence slit with angular range of 5. The samples were placed in an aluminium sample holder and continuously scanned from 5° to 45° range of 20 (with a step size of 0.017° 0/min and step time of 25 sec/step) with a scan rate of 3°/min. The obtained data was analyzed using X’PERT high Score software.

4.7.3 Fourier Transform Infrared (FTIR) Spectroscopy
FTIR spectrometer, Spectrum RX II (Perkin Elmer, England), installed in University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh was used for obtaining the FTIR spectra. The samples were mixed with potassium bromide (KBr) and pellets were prepared. The spectrum was taken from 4 accumulative scans in the transmittance range of 400–4000 cm\(^{-1}\) and at a spectral resolution of 4 cm\(^{-1}\). The data was analyzed using Spectrum software.

4.7.4 Solid State Nuclear Magnetic Rasonance (SSNMR)
This facility was availed from NMR research centre, Indian Institute of Science (IISC), Bengaluru. Joel Resonance 400 MHz spectrometer (Joel, Massachusetts, USA) operating at resonant frequency of 100 MHz was used for recording solid state \(^{13}\)C NMR spectra. SSNMR measurements were carried out on 4 mm double resonance CP/MAS probe, spinning at a rate of 10 KHz with a cross polarization contact time of 3.5 ms and relaxation delay of 5 seconds. The data was collected at 273 K to minimize frictional heating effects. The data was analyzed using Delta TM NMR software. The spectra were referred to methylene carbon of glycine (\(\delta\) glycine= 43.3 ppm) and then chemical shifts were recalculated to tri methyl silane (TMS).

4.8 Crystal structure determination from PXRD
The PXRD patterns of the cocrystals and polymorphs were subjected to Material Studio® software 7.0 (BIOVIA) to determine the crystal structures. This software is available in University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh. The elucidation of the crystal structure was done in four steps i.e., Indexing, Pawley refinement, Powder solve and Rietveld refinement. The PXRD patterns were preprocessed and prepared for indexing by subtracting the background, smoothening of peaks followed by stripping.

Powder Indexing
Indexing of the peak positions in the experimental powder diffraction pattern was done utilizing X-cell (Neuman, 2003) module from which appropriate crystal lattices were obtained. From the solutions, a unit cell with maximum figure of merits was selected for further step.
Pawley Refinement
The unit cell obtained from indexing was refined by pawley refinement (Pawley, 1981). The unit cell was optimized by 10 cycles and then it was searched for space groups. Again a unit cell with appropriate space group was created and subjected to pawley refinement with the same conditions as followed in the previous refinement.

Powder Solve
The geometrically optimized structures of APIs and respective coformers (the optimization of geometry of the molecules was done using DMOL$^3$) were imported in the refined unit cell and subjected to powder solve (Engel et al., 1999) module with 10 simulated annealing cycles having 100000 iterations in each cycle.

Rietveld Refinement
The structure of cocrystal obtained after powder solve was refined by rietveld refinement (Rietveld, 1969; Young, 1993). The obtained Rwp (weighted Rietveld parameter) after refinement depicts similarity between the experimental and the calculated diffraction patterns. Again the crystal structure was optimized with the forcite module and then exported in the cif format for analysis.

4.9 High Performance Liquid Chromatography (HPLC)
For the quantitative estimation of GL, GPZ and RPG, the methods were developed and validated on Waters HPLC system (USA), equipped with LC-10AT pump and PDA detector (Waters 2996). The chromatographic separation of the analytes was performed using Thermofisher C$_{18}$ column (4.6 mm x 250 mm, 5μm) and Empower 2 software was used for the acquisition and analysis of the HPLC data.

Gliclazide: A method consisting of isocratic mobile phase of ACN and buffer (water of pH 3.0; pH was adjusted with orthophosphoric acid) in the 50:50 ratio with a flow rate of 1.2 mL/min and injection size of 10 μL was chosen for the determination of GL through HPLC. Prior to the injection, the column was equilibrated for at least 30 minutes with mobile phase. The peak of GL was detected at 228 nm with retention time 5.8 min. The method was validated by measuring the validation parameters such as linearity, accuracy, inter-day or intra-day precision, limit of detection and limit of quantification. For the calibration curve, the standard stock solution of GL (100 μg/mL) was prepared in ACN and further serial dilutions were done by mobile phase in the concentration range 0.05-100 μg/mL. The resultant samples were filtered through a 0.45 μm membrane filter for removing any
particulate matter. The same method was followed for the detection of GL in plasma. The different concentrations of GL were spiked (0.05-100 µg/mL) in plasma. Following the extraction procedure, the calibration curves of GL in plasma were prepared. GL was detected at the same wavelength \( i.e., \) 228 nm without any shift in retention time.

**Glipizide:** After a number of trials, the optimum separation of GPZ was seen from the mobile phase of ACN and buffer (water of pH 3.0; pH was adjusted with orthophosphoric acid) in the 50:50 ratio with a flow rate of 0.8 mL/min. 10 µL sample was injected into the column and prior to the injection, the column was equilibrated for atleast 30 minutes with mobile phase. The peak of GPZ was detected at 227 nm with retention time 6.6 min. The method was validated by measuring the validation parameters such as linearity, accuracy, inter-day or intra-day precision, limit of detection and limit of quantification. For the calibration curve, the standard stock solution of GPZ (100 µg/mL) was prepared by dissolving 5 mg of GPZ in 50 mL of ACN and further serial dilutions were done by mobile phase in the concentration range 0.05-100 µg/mL. The resultant samples were filtered through a 0.45 µm membrane filter for removing any particulate matter. The same method was followed for the detection of GPZ in plasma. The different concentrations of GPZ were spiked (0.05-100 µg/mL) in plasma. Following the extraction procedure, the calibration curves of GPZ in plasma were prepared. GPZ was detected at the same wavelength \( i.e., \) 227 nm without any shift in retention time.

**Repaglinide:** A method consisting of isocratic mobile phase of ACN and buffer (water of pH 3.0; pH was adjusted with orthophosphoric acid) in the 50:50 ratio with a flow rate of 0.8 mL/min and injection size of 10 µL was chosen for the determination of RPG through HPLC. Prior to the injection, the column was equilibrated for at least 30 minutes with mobile phase. The peak of RPG was detected at 230 nm with retention time 6.7 min. The method was validated by measuring the validation parameters such as linearity, accuracy, inter-day or intra-day precision, limit of detection and limit of quantification. For the calibration curve, the standard stock solution of RPG (100 µg/mL) was prepared in ACN and further serial dilutions were done by mobile phase in the concentration range 0.05-100 µg/mL. The resultant samples were filtered through a 0.45 µm membrane filter for removing any particulate matter. The same method was followed for the detection of RPG in plasma. The different concentrations of RPG were spiked (0.05-100 µg/mL) in plasma. Following the extraction procedure, the calibration curves of RPG in plasma were prepared. RPG was detected at the same wavelength \( i.e., \) 230 nm without any shift in retention time.
4.10 Equilibrium solubility and intrinsic dissolution studies of cocrystals

4.10.1 Equilibrium solubility studies
The equilibrium solubility study of the GL, GPZ, RPG and its cocrystals were performed by the method reported by Higuchi and Conners (Higuchi and Conners, 1965) i.e., shake flask method. An excess amount of GL, GPZ, RPG and its cocrystals (approximately 20 mg) was added in a vial containing 5 mL of buffer (for GL, GPZ and their cocrystals: phosphate buffer of pH 7.4; for RPG and its cocrystals: citrate buffer of pH 5.0). The vials were agitated for 24 h using water bath shaker, MSW-275 (Macroscientific Works, Delhi) with 200 rpm at 37 °C. After vigorous shaking, the resulting slurry was filtered through a 0.45 µm membrane filter and the estimation of the released amount of GL, GPZ, and RPG from respective cocrystals in the buffer was determined by HPLC. The data of the equilibrium solubility was expressed in ± SD values.

4.10.2 Intrinsic dissolution studies
Intrinsic dissolution studies of GL, GPZ, RPG and its cocrystals were performed using rotating disk dissolution test apparatus, DS 8000 (Lab India Analyticals) at 37 °C for 4 h. 500 mL phosphate buffer of pH 7.4 was used as dissolution media for GL, GPZ (recommended in USP) and their cocrystals whereas 500 mL citrate buffer of pH 5.0 was used as dissolution media for RPG and its cocrystals (recommended in USP). 150 mg of sample was put in a die and the punch was compressed by benchtop carver press for 1-2 min at 2000 psi. The base plate was then disconnected from the die to expose a smooth compact pellet with a 0.5 cm² surface area. Neoprene gasket was placed around the threaded shoulder of the die which was then screwed onto the shaft holder and the shaft was mounted on the stirring hood of the dissolution apparatus. After mounting, the dies were lowered into the dissolution vessel containing dissolution media. Aliquot samples (5 mL) were withdrawn (with replacement) at specified time interval (5, 10, 15, 30, 45, 60, 90, 120, 180, 240 min), filtered through 0.45 µm membrane filter and quantitatively analyzed by HPLC. The obtained IDR of APIs in respective cocrystals (by analyzing the slope of a graph between respective concentrations vs. time) was compared with that of pure drug. The data of IDR was expressed in ± SD values.

4.11 In-vivo studies of cocrystals
For the pharmacokinetic and pharmacodynamic study, male Wistar rats (3-4 weeks old; 150-200 g) were procured and retained in Central Animal House for adaptation of environment. The animals were provided with standard pellet diet and water ad libitum. Experiments were performed as per guidelines of CPCSEA (Committee for the Purpose of the Control and
Supervision on Experiments on Animals). The experimental protocol was approved by Institutional Animal Ethics Committee (I.A.E.C.) under approval no- PU/IAEC/S/14/70.

4.11.1 Pharmacokinetic study
The pharmacokinetic activity of GL was performed on normal rats whereas of GPZ and RPG were performed in diabetic male Wistar rats. The sampling of the blood was done at specified intervals of time and the processed plasma was analyzed by HPLC. The pharmacokinetic parameters such as peak plasma concentration ($C_{\text{max}}$), time of peak concentration ($T_{\text{max}}$) and area under the curve at time $t$ ($\text{AUC}_{0-t}$) were determined by PKSolver: An Add–in program (Zhang et al., 2010) which does calculation based on linear trapezoidal method.

4.11.1.1 Sample preparation
The blood samples were centrifuged at 5000 rpm for 3 min to separate plasma. From the 100 µL of plasma, the respective API was extracted to ACN by adding 900 µL ACN, vortexed for 2 min and again centrifuged at 10,000 rpm for 15 min. 800 µL plasma from the top was taken and analyzed for API content with the help of HPLC.

4.11.1.2 Protocol of pharmacokinetic studies
**GL and its cocrystals:** A single dose of GL and its cocrystals (equivalent to 40 mg/kg of GL, suspended in normal saline) was administered orally to the respective test groups of normal rats (Talari et al., 2010). The dose of GL and its cocrystals, administered to rats is given in table 4.3. The sampling was done for 24 h at different intervals of time (0.5, 1, 2, 4, 8, 10, 12, 24 h). The plasma samples were analyzed quantitatively by HPLC.

The rats were divided into following six groups and each group comprised six rats (n=6).

- **Group I:** Control group; normal saline (vehicle) was administered to the normal rats.
- **Group II:** Standard group; GL suspended in normal saline was administered to the normal rats.
- **Group III:** Test group; GL-SA suspended in normal saline was administered to the normal rats.
- **Group IV:** Test group; GL-HA suspended in normal saline was administered to the normal rats.
- **Group V:** Test group; GL-SB suspended in normal saline was administered to the normal rats.
- **Group VI:** Test group; GL-MA suspended in normal saline was administered to the normal rats.

**GPZ and its cocrystals:** A single dose of GPZ and its cocrystals (equivalent to 5 mg/kg of GPZ; suspended in 2% sodium CMC) was administered orally to the respective test groups of diabetic rats (Babu et al., 2012). The dose of GPZ and its cocrystals, administered to rats is given in table 4.3. The sampling was done for 8 h at different intervals of time (0.5, 1, 2, 3, 4, 5, 6 and 8 h). The plasma samples were analyzed quantitatively by HPLC.
The rats were divided into following seven groups and each group comprised six rats (n=6).

Group I: Diabetic control group; 2% sodium CMC (vehicle) was administered to the diabetic rats.

Group II: Standard group; GPZ suspended in 2% sodium CMC was administered to the diabetic rats.

Group III: Test group; GPZ-PA suspended in 2% sodium CMC was administered to the diabetic rats.

Group IV: Test group; GPZ-AA suspended in 2% sodium CMC was administered to the diabetic rats.

Group V: Test group; GPZ-INA suspended in 2% sodium CMC was administered to the diabetic rats.

Group VI: Test group; GPZ-FA suspended in 2% sodium CMC was administered to the diabetic rats.

Group VII: Test group; GPZ-SRA suspended in 2% sodium CMC was administered to the diabetic rats.

**RPG and its cocrystals:** A single dose of RPG and its cocrystals (equivalent to 0.18 mg/kg of RPG; suspended in 1.5% methylcellulose) was administered orally to the respective test groups of diabetic rats (Mark and Grell, 1997; Masiello et al., 1998; Sekhar and Reddy, 2012). The dose of RPG and its cocrystals, administered to rats is given in table 4.3. The sampling was done for 8 h at different intervals of time (0.5, 1, 2, 3, 4, 5, 6 and 8 h). The plasma samples were analyzed quantitatively by HPLC.

**Table 4.3: Dose of GL, GPZ, RPG and their cocrystals, administered to rats for pharmacokinetic study**

<table>
<thead>
<tr>
<th>GL and its cocrystals</th>
<th>Dose (mg/kg); equivalent to 40 mg/kg of GL</th>
<th>GPZ and its cocrystals</th>
<th>Dose (mg/kg); equivalent to 5 mg/kg of GPZ</th>
<th>RPG and its cocrystals</th>
<th>Dose (mg/kg); equivalent to 0.18 mg/kg of RPG</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL</td>
<td>40</td>
<td>GPZ</td>
<td>5</td>
<td>RPG</td>
<td>0.18</td>
</tr>
<tr>
<td>GL-SA</td>
<td>54.61</td>
<td>GPZ-PA</td>
<td>6.38</td>
<td>RPG-HA</td>
<td>0.21</td>
</tr>
<tr>
<td>GL-HA</td>
<td>49.41</td>
<td>GPZ-AA</td>
<td>6.64</td>
<td>RPG-PA</td>
<td>0.23</td>
</tr>
<tr>
<td>GL-SB</td>
<td>65.01</td>
<td>GPZ-INA</td>
<td>6.38</td>
<td>RPG-SB</td>
<td>0.26</td>
</tr>
<tr>
<td>GL-MA</td>
<td>56.58</td>
<td>GPZ-FA</td>
<td>6.30</td>
<td>RPG-AA</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GPZ-SRA</td>
<td>6.26</td>
<td>RPG-PD</td>
<td>0.25</td>
</tr>
</tbody>
</table>
The rats were divided into following seven groups and each group comprised six rats (n=6).

Group I: Diabetic control group; 1.5% methylcellulose (vehicle) was administered to the diabetic rats.

Group II: Standard group; RPG suspended in 1.5% methylcellulose was administered to the diabetic rats.

Group III: Test group; RPG-HA suspended in 1.5% methylcellulose was administered to the diabetic rats.

Group IV: Test group; RPG-PA suspended in 1.5% methylcellulose was administered to the diabetic rats.

Group V: Test group; RPG-SB suspended in 1.5% methylcellulose was administered to the diabetic rats.

Group VI: Test group; RPG-AA suspended in 1.5% methylcellulose was administered to the diabetic rats.

Group VII: Test group; RPG-PD suspended in 1.5% methylcellulose was administered to the diabetic rats.

4.11.1.3 Pharmacokinetic statistical analysis
The pharmacokinetic parameters were represented by mean ± SD and compared with control group by One-way ANOVA followed by Dunnett’s test and Student’s test using GraphPad Prism 6.0 software at 95% confidence interval.

4.11.2 Pharmacodynamic study
The diabetes was induced by injecting a single dose of the solution of streptozotocin (STZ) with nicotinamide (45 mg/kg; prepared in citrate buffer of pH 4.4, 0.1 M) (Zafar et al., 2009) via intraperitoneal route. The rats were found diabetic after 48 h of injection. For the assessment of antidiabetic activity, GL, GPZ, RPG and their cocrystals were administered to the diabetic male Wistar rats and the reduction in the plasma glucose level was monitored.

4.11.2.1 Sampling of plasma
The blood samples from retro-orbital plexus of the rats were withdrawn according to the protocol and centrifuged at 5,000 rpm for 3 min to separate the plasma from blood. Then one drop of acetonitrile was added (to precipitate proteins) to plasma, vortexed it for 2 min and again centrifuged at 10,000 rpm for 15 min. The concentration of glucose in protein free plasma was checked by Erba glucose kit (Transasia Biomedical Pvt. Ltd., Solan), which is based on trinder’s methodology and the end point is determined by enzymatic GOD – POD (glucose oxidase peroxidase) method.
4.11.2.2 Analysis of glucose by GOD – POD method (Trinder, 1969)

Glucose present in the plasma forms gluconic acid in the presence of enzyme glucose oxidase which in turn reacts with 4-Hydroxy benzoic acid (4HBA) and 4-Aminoantipyrine (4AAP) in the presence of enzyme peroxidase to form quinoneimine dye (pink colour).

\[
\text{Glucose} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{glucose oxidase}} \text{Gluconic acid} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + 4\text{HBA} + 4\text{AAP} \xrightarrow{\text{peroxidase}} \text{Quinoneimine Dye} + 2\text{H}_2\text{O}_2
\]

The intensity of the formed pink colour is proportional to the glucose concentration.

For the estimation of glucose by this method, the solutions were prepared as per following scheme (Table 4.4)

Table 4.4: Method of preparation of solutions for pharmacodynamic study

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Working reagent</strong></td>
<td>1000μl</td>
<td>1000μl</td>
<td>1000μl</td>
</tr>
<tr>
<td><strong>Distilled water</strong></td>
<td>10μl</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>Standard</strong></td>
<td>---</td>
<td>10μl</td>
<td>---</td>
</tr>
<tr>
<td><strong>Test</strong></td>
<td>---</td>
<td>---</td>
<td>10μl</td>
</tr>
</tbody>
</table>

Absorbance of standard and each test solution prepared by following above scheme against reagent blank was noted at 505 nm by using Lambda 25 UV/VIS spectrometer and glucose concentration was calculated from the following formula –

\[
\text{Glucose (10mg/mL)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{concentration of standard (mg/dL)}
\]

4.11.2.3 Protocol of antidiabetic activity

**GL and its cocrystals:** To study the efficacy of GL and its cocrystals in reducing the plasma glucose level, a single dose of GL and of four prepared cocrystals (equivalent to 45 mg/kg of GL; suspended in citrate buffer of pH 4.4, 0.1 M) was administered orally to the respective test groups of Wistar rats (Talari et al., 2010). The dose of GL and each cocrystal which was administered to rats is given in table 4.5.

After seven days the blood samples were withdrawn, treated as described in section 4.11.2.1 and analyzed for glucose level reduction.

For the study, the rats were divided into six groups and each group comprised six rats (n=6).

Group I: Diabetic control group; citrate buffer pH 4.4 (vehicle) was administered to the diabetic rats.
Group II: Standard group; GL suspended in citrate buffer pH 4.4 was administered to the diabetic rats.

Group III: Test group; GL-SA suspended in citrate buffer pH 4.4 was administered to the diabetic rats.

Group IV: Test group; GL-HA suspended in citrate buffer pH 4.4 was administered to the diabetic rats.

Group V: Test group; GL-SB suspended in citrate buffer pH 4.4 was administered to the diabetic rats.

Group VI: Test group; GL-MA suspended in citrate buffer pH 4.4 was administered to the diabetic rats.

**GPZ and its cocrystals:** A single dose of GPZ and its cocrystals (equivalent to 5 mg/kg of GPZ; suspended in 2% sodium CMC) were administered orally to the respective test groups of Wistar rats (Babu et al., 2012). The dose of GPZ and each cocrystal which was administered to rats is given in table 4.5.

The extent of reduction in blood glucose was measured for 8 h at different intervals of time (0.5, 1, 2, 3, 4, 5, 6 and 8 h). For this study, the rats were divided into following seven groups and each group comprised six rats (n=6).

Group I: Diabetic control group; 2% sodium CMC (vehicle) was administered to the diabetic rats.

Group II: Standard group; GPZ suspended in 2% sodium CMC was administered to the diabetic rats.

Group III: Test group; GPZ-PA suspended in 2% sodium CMC was administered to the diabetic rats.

Group IV: Test group; GPZ-AA suspended in 2% sodium CMC was administered to the diabetic rats.

Group V: Test group; GPZ-INA suspended in 2% sodium CMC was administered to the diabetic rats.

Group VI: Test group; GPZ-FA suspended in 2% sodium CMC was administered to the diabetic rats.

Group VII: Test group; GPZ-SRA suspended in 2% sodium CMC was administered to the diabetic rats.

**RPG and its cocrystals:** A single dose of RPG and its cocrystals (equivalent to 0.18 mg/kg of RPG; suspended in 1.5% methylcellulose) were administered orally to the respective test groups of Wistar rats (Mark and Grell, 1997; Masiello et al., 1998; Sekhar and Reddy, 2012). The dose of RPG and each cocrystal which was administered to rats is given in table 4.5.
Table 4.5: Dose of GL, GPZ, RPG and their cocrystals, administered to rats for pharmacodynamic study

<table>
<thead>
<tr>
<th>GL and its cocrystals</th>
<th>GL</th>
<th>45</th>
<th>GPZ</th>
<th>5</th>
<th>RPG</th>
<th>0.18</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL-SB</td>
<td>61.63</td>
<td>GPZ-PA</td>
<td>6.38</td>
<td>RPG-HA</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>GL-HA</td>
<td>55.58</td>
<td>GPZ-AA</td>
<td>6.64</td>
<td>RPG-PA</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>GL-SB</td>
<td>73.14</td>
<td>GPZ-INA</td>
<td>6.38</td>
<td>RPG-SB</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>GL-MA</td>
<td>63.66</td>
<td>GPZ-FA</td>
<td>6.30</td>
<td>RPG-AA</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GPZ-SRA</td>
<td>6.26</td>
<td>RPG-PD</td>
<td>0.25</td>
<td></td>
</tr>
</tbody>
</table>

The extent of reduction in blood glucose was measured for 6 h at different intervals of time (0.25, 0.5, 1, 1.5, 2, 3, 4, and 6 hrs). The rats were divided into following seven groups and each group comprised six rats (n=6).

Group I: Diabetic control group; 1.5% methylcellulose (vehicle) was administered to the diabetic rats.

Group II: Standard group; RPG suspended in 1.5% methylcellulose was administered to the diabetic rats.

Group III: Test group; RPG-HA suspended in 1.5% methylcellulose was administered to the diabetic rats.

Group IV: Test group; RPG-PA suspended in 1.5% methylcellulose was administered to the diabetic rats.

Group V: Test group; RPG-SB suspended in 1.5% methylcellulose was administered to the diabetic rats.

Group VI: Test group; RPG-AA suspended in 1.5% methylcellulose was administered to the diabetic rats.

Group VII: Test group; RPG-PD suspended in 1.5% methylcellulose was administered to the diabetic rats.

4.11.2.4 Pharmacodynamic statistical analysis

The data of percentage of glucose reduction from APIs and respective cocrystals was represented by mean ± SD and compared with control group by One-way ANOVA followed by Dunnett’s test and Student’s test using GraphPad Prism 6.0 software at 95% confidence interval.