CHAPTER - V

DEVELOPMENT AND VALIDATION OF ANALYTICAL METHOD FOR GLIPIZIDE
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Development of analytical method is important part of any research work. The analytical methods are required for quantitative determination of drug in the formulation, skin layers, release medium and also in blood. The analytical method should be specific, accurate, sensitive and reproducible for the drug.

Several procedures have been developed and reported to be used as standard methods for the analysis of sulfonylureas.

UV-Spectrophotometric method is official in United State Pharmacopoeia XXIV(1999) based on the measurement of absorbance at 223 nm and 276 nm.

Emilsson H. (1987) had reported reverse phase HPLC method for analysis of Glipizide in human plasma and urine with phosphate buffer (pH3.5) and acetonitrile in 65:35 as a mobile phase. The retention time of Glipizide was found to be 6.67 min.

Strausbauch et al (1995) developed the method for concentration and separation of hypoglycemic drugs using solid-phase extraction-capillary electrophoresis. Study included the development of a method exploiting the technique for the direct analysis of hypoglycemic drugs in urine. The method was developed for effective separation and detection of six sulfonylurea drug standards at concentrations below the detection limit of conventional capillary electrophoretic techniques.

Hans et al (2002) developed an atmospheric pressure chemical ionization liquid chromatographic–mass spectrometric (APCI–LC–MS) LC–MS assay for sulfonylureas. This method was found to be fast and reliable for screening and identification as well as precise and sensitive for quantification of oral anti-diabetics of the sulfonylurea type in plasma. It allowed the specific diagnosis of an overdose situation or a Munchausen’s syndrome caused by ingestion of sulfonylurea.
Emmie et al (2004) described a convenient method for the separation and simultaneous detection of 10 anti-diabetic drugs (namely Glipizide, glibenclamide, glimeperide, gliclazide, tolazamide, tolbutamide, nateglinide, repaglinide, rosiglitazone and pioglitazone) in equine plasma and urine by LC–MS–MS. The LODs for all 10 anti-diabetics were well below 1 ng/mL.

Zhongping et al (2004) reported a method to investigate drug–drug protein binding interaction between Glipizide and rosiglitazone. The method was developed and validated for simultaneously determining the free (unbound) fraction of Glipizide and rosiglitazone in plasma employing equilibrium dialysis for the separation of free drug and liquid chromatography–tandem mass spectrometry (LC–MS/MS) for quantitation. The chromatographic run time was 5 min per injection, with retention times of 2.3 and 3.4 min for Glipizide and rosiglitazone respectively. The calibration curves of Glipizide and rosiglitazone were linear over the range of 1–2000 ng/ml ($r^2 > 0.9969$) in the combined matrix of human plasma and isotonic sodium phosphate buffer (1:1, v/v). The lower limit of quantitation of both Glipizide and rosiglitazone was 1.0 ng/ml.

AbuRuz et al (2005) developed the solid phase extraction and HPLC methods for the simultaneous determination of metformin and Glipizide, gliclazide, glibenclamide or glimeperide in plasma. The validity, LOQ and linearity range of the method makes it an acceptable method for clinical studies in diabetes patients taking these medications. The LOQ and LOD for Glipizide by this method were found to be 7.5 and 4.5 ng/ml respectively.

Jing Yao et al (2007) developed an isocratic reversed-phase high performance liquid chromatography (RP-HPLC) method for screening counterfeit medicines and adulterated dietary supplement products. The developed method could be employed to
separate and determine simultaneously six anti-diabetic drugs (Glipizide, gliclazide, glibenclamide, glimepiride, gliquidone, repaglinide) on an isocratic solvent system using an Allitina C18 column (5-m, 150mm×4.6 mm) with an isocratic mobile phase of methanol-phosphate buffer (pH 3.0; 0.01 mol/L) (70:30, v/v), at a flow rate of 1.0 ml/min and at a wavelength of 230 nm.

Ghoneim et al (2007) used the electrochemical behavior of Glipizide at the hanging mercury drop electrode for stripping voltammetric quantification of the anti-diabetic drug Glipizide in bulk form and pharmaceutical formulation. The proposed procedure was successfully applied for quantitation of Glipizide in its pharmaceutical formulation without interference from excipients.

5.1 UV- Visible spectroscopy method development

The UV absorption spectrum of 50 μg/ml of Glipizide solution in phosphate buffer (7.4 pH) obtained by using double beam UV visible spectrophotometer (Schimadzu-UV 2501PC), revealed two absorption maxima at 230 nm and 276 nm (λ_{max}) for Glipizide.

Stock solution was prepared by dissolving 10 mg of Glipizide in 100 ml of phosphate buffer (pH 7.4) to obtain the solution of 100μg/ml. From this stock solution, suitable amount of aliquots were transferred into a series of 10 ml volumetric flasks and made up to volume with phosphate buffer to obtain the solutions of 2, 4, 6, 8 μg/ml concentrations. Absorbance of theses solutions was measured by UV visible spectrophotometer (Schimadzu- UV 2501PC) at 230 nm and plotted against the concentration to give standard curve (Fig: 5.1).
All the samples were found to obey Beer's law for linearity and correlation of coefficient was found to be 0.9975.

5.2 Reverse Phase HPLC method development

The literature survey revealed many HPLC methods for analysis of Glipizide. United state pharmacopoeia describes an official reverse phase HPLC method for analysis of Glipizide in pharmaceutical dosage form and bulk. The mobile phase recommended is Phosphate buffer (pH 6.00 ± 0.05) and methanol in 55:45 ratios. The wave length is 225 and column size 15 cm × 3.9 cm with flow rate of 1.00 ml/min. Sethi P.D., described the reverse phase HPLC method for analysis of Glipizide at wavelength 230nm with a mobile phase potassium acid Phosphate (0.05 M) and methanol (40:60 v/v) adjusted to pH 3.00. The C-18 column (250 × 4.6 mm) was used with flow rate of 1ml/min. The retention time reported was 6.3 min and linearity range was 0.4 to 10 μg/ml.

The method reported by Sethi P.D. had retention time of 6.3 min and flow rate 1ml/min. This makes the analysis of Glipizide time consuming and costly because of high solvent consumption.
Hence a new method was developed with intention to reduce retention time, requiring less mobile phase and time. The developed method was validated for linearity, accuracy and precision.

5.2.1 Instrument specifications

Instrument: Dionex-UVD170U

Column: PC-Micra NPS RP18, (length × OD × ID = 33 × 8.0 × 4.6 mm, 1.5 μm)

Pump: P 680 HPLC

Sample injector: ASI automated sample injector

Data acquisition and processing system software: Chromelone (Chromatography Information management System)

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) using filtration assembly with vacuum pump (Rocker pump 400, Today’s) and ultrasonicated using ultrasonic water bath (UCB 100, Spectralab) for degassing.

5.2.2 Preparation and optimization of Mobile phase

Different ratios of phosphate buffer and methanol were tried as mobile phase to get optimal retention time and other peak parameters. The pH of mobile phase was optimized by trying the mobile phase of different pH. The detector was set at wavelength of 226 nm and response peaks were integrated using software.

The pH of mobile phase optimized by trying three different pH 3.00, 4.5, and 7.4. Required quantity of monobasic potassium di-hydrogen phosphate (HPLC grade) (KH₂PO₄) was dissolved in 1000 ml of HPLC grade water. The pH was adjusted with O-phosphoric acid. The buffer and HPLC grade methanol was filtered through
0.22μm nylon filter (Millipore) and then sonicated for 10 min to make it free from dissolved gases.

The composition and pH of mobile phase was optimized by several preliminary experimental trials to achieve good peak symmetry and short retention time (RT). Optimization of various conditions was done by varying one parameter at a time and keeping other parameters constant. The effect on peak shape and RT were the observation parameters. At pH 3.00 the RT was higher, as the pH of mobile phase increased to 7.4 the peak shows trailing which increases peak asymmetry. The optimum pH was found to be 4.5, at which symmetric peak was obtained with the retention time of 4.1 min. During optimization it was observed that as the amount of methanol increased the RT decreases. The optimal ratio of phosphate buffer (pH4.5) and methanol was found to be 40:60. At optimized ratio the peak was symmetric and retention time was 1.89 min, which was much lesser than the reported method. Under this experimental condition, the chromatogram of Glipizide was shown in Fig 5.2.

5.2.3 Chromatogram of Glipizide

![Chromatogram of Glipizide (100 μg/ml)](image)

Fig. 5.2: Chromatogram of Glipizide (100 μg/ml)
5.2.4 Flow rate optimization
Optimization of flow rate was also carried out by trying various flow rates between 0.1 ml/min to 0.5 ml/min. The volume of injection was 10 μl. The optimal flow rate was found to be 0.4 ml/min.

5.2.5 Preparation of standard solutions and construction of Calibration curve
The stock solution of Glipizide 1mg/ml was prepared by dissolving accurately about 25 mg of Glipizide in 25 ml of HPLC grade methanol. The stock solution was further diluted serially with mobile phase to get the solutions of concentration in the range 50 to 200 μg/ml. The samples were filtered through 0.22μm nylon filter (Millipore) before analysis. 10μl of each of the standard solution was injected and peak area obtained. A calibration curve was constructed by plotting peak areas versus concentrations. (Fig.5.3)

![Graph showing calibration curve](image)

<table>
<thead>
<tr>
<th>No.</th>
<th>Ret.Time (min)</th>
<th>Peak Name</th>
<th>Cal.Type</th>
<th>Points</th>
<th>Coeff.Det. (%)</th>
<th>Offset</th>
<th>Slope</th>
<th>Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>2</td>
<td>1.89</td>
<td>Glu</td>
<td>Lin</td>
<td>4</td>
<td>99.5033</td>
<td>0.0000</td>
<td>1.0378</td>
<td>0.0000</td>
</tr>
<tr>
<td>Average</td>
<td>99.5033</td>
<td>0.0000</td>
<td>1.0378</td>
<td>0.0000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 5.3: Standard calibration curve for Glipizide (RP-HPLC)
A four point calibration curve was constructed and found to be linear. 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 0.9950.

5.3 Method validation

The analytical performance parameters of the method such as linearity, precision and accuracy, limit of detection and limit of quantification were validated according to International Conference on Harmonization (ICH Q2B) guidelines.

5.3.1 Linearity, limit of quantification and limit of detection

Four different concentration levels (50 to 200μg/ml) were obtained from the stock solution by diluting with the mobile phase. Each concentration solution was prepared in triplicate. The samples were analyzed and graph was plotted between the average peak area and the corresponding concentrations. The equations of linear regression were performed using least-squares method.

The limit of quantification (LOQ) was the lowest concentration assayed where the signal/noise ratio was at least 10:1. The limit of detection (LOD) was defined as a signal/noise ratio of 3:1. Based on the peak area from the diluted solution of drug in the sample, the LOD and LOQ were calculated from following equations.

$$LOD = 3.3 \times \text{S.D.} / \text{Slope}$$
$$LOQ = 10 \times \text{S.D.} / \text{Slope}$$

The linearity of the method used was evaluated by construction of a standard curve (Fig. 5.3) of the average peak area (y) versus the concentration of the Glipizide (x, μg/ml). Each dilution (50 to 200μg/ml) was prepared in triplicate. A four point calibration curve was constructed and found to be linear ($r^2 = 0.9997$). The slope was calculated using the plot of drug concentration versus average peak area of the chromatogram. The LOD and LOQ of the method were found to be 0.10 and 0.75 μg/ml respectively.
5.3.2 Accuracy and precision

Three replicates of three different concentrations (10, 50, 100 μg/ml) of the sample solutions were analyzed on three different days in order to determine the accuracy and precision. One-way analysis of variance (ANOVA) was used to calculate the intra- and inter-day variation in these parameters.

Table 5.1: Determination of accuracy and precision.

<table>
<thead>
<tr>
<th>Spiked Concentration (μg/ml)</th>
<th>Measured concentration (μg/ml)</th>
<th>Accuracy (%)</th>
<th>Intra-day precision (%)</th>
<th>Inter-day Precision (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>9.95</td>
<td>99.5</td>
<td>0.16</td>
<td>0.42</td>
</tr>
<tr>
<td>50</td>
<td>49.87</td>
<td>99.74</td>
<td>0.20</td>
<td>0.26</td>
</tr>
<tr>
<td>100</td>
<td>99.6</td>
<td>99.6</td>
<td>0.10</td>
<td>0.32</td>
</tr>
</tbody>
</table>

These results showed that the method was accurate (accuracy >99.0%) and precise (intra-day precision from 0.10% to 0.20% and inter-day precision from 0.26% to 0.42%).

5.3.3 System suitability tests

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

Table 5.2: 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>1.31 ± 0.003</td>
<td>Complies</td>
</tr>
<tr>
<td>Theoretical plates (&gt; 2000)</td>
<td>278 ± 4</td>
<td>*</td>
</tr>
<tr>
<td>RSD (USP: RSD&lt; 2.0)</td>
<td>0.35 %</td>
<td>Complies</td>
</tr>
</tbody>
</table>

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

System suitability parameters such trailing/ asymmetry factor and relative standard deviation (RSD) were found to be well within acceptable limits (Table 5.2). But the number of theoretical plates observed lesser than the recommended value which may be due to the short length of column (33mm).
5.3.4 Robustness

The robustness was evaluated by deliberate variations of the method parameters. The factors selected to examine were flow rate (ml/min), pH of mobile phase, temperature (°C). One factor at a time was changed to estimate the effect. Deliberate alteration in the parameters included, the mobile phase flow rate in the variants: 0.3 and 0.5 mL/min, the pH of buffer in the variants: 4.4 and 4.6 and the temperature in the variants: 27 and 33°C. The retention time and peak parameters of each variation were evaluated. No significant changes were occurred in peak parameters and RT under the deliberately modified conditions which proved robustness of the method.

The newly developed RP-HPLC method was found to be a simple, accurate, less time consuming and economical for the analysis of Glipizide in the sample solution. Thus this method was used for the analysis of all the samples in further research work.

5.4 Summary and conclusion

The new RP-HPLC method developed had high degree of accuracy, precision and specificity. After trying various combinations of mobile phase components with various pH ranges, the optimal ratio of phosphate buffer (pH 4.5) and methanol was found to be 40:60. At optimized ratio the peak was symmetric and retention time was 1.89 min, which was much lesser than earlier reported method. The method was validated for system suitability tests, linearity, robustness, limit of quantification and limit of detection. The developed method requires less volume of mobile phase, less retention time and hence economical. This method was used for further research work.
5.5 References


