RESEARCH ARTICLE

DETERMINATION OF METFORMIN IN RAT PLASMA USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS APPLICATION TO PHARMACOKINETIC STUDY

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ARTICLE INFO

Article History:
Received 27th March, 2016
Received in revised form 23rd April, 2016
Accepted 04th May, 2016
Published online 30th June, 2016

Keywords:
Metformin, Phenformin, Rat plasma, Pharmacokinetics.

ABSTRACT

A rapid and sensitive HPLC-PDA method was developed for the quantification of metformin in rat plasma using phenformin as internal standard. The analyte and internal standard were extracted from the plasma sample using a solid phase extraction method. Chromatographic separation was achieved on a Thermo Accucore Hillic (150 × 4.6 mm, 5 µm) column with a mobile phase consisting of 20 mM sodium acetate buffer and acetonitrile (45:55, v/v with pH 4); at a flow rate of 0.7 ml /min. The assay was validated with a linear range of 0.062 – 2 µg /ml for metformin using 200 µL of plasma sample. The intra- and inter-day assay precision ranged from 2.51 to 4.37% and 2.19 to 5.59%, respectively, and intra- and inter-day assay accuracy was between from 0.27 to 3.35% and -0.51 to 3.75%, respectively. The method was successfully applied to the pharmacokinetic studies of metformin in rat plasma.

INTRODUCTION

The worldwide rise in type 2 diabetes presents a major public health problem. In 2011, there were 366 million people with type 2 diabetes, a number projected to rise up to 552 million by 2030 (Whiting, 2011) This corresponds to an annual growth of 2.7%, outstripping by a factor of 1.7 in respect of the global population (Whiting, 2011) This increase in prevalence will disproportionately affect developing countries which are least able to cope with medical and economic consequences for example, 48% of increase in prevalence is projected to occur in India and China (Whiting, 2011). In India the increase is estimated to be 58%, from 51 million people in 2010 to 87 million in 2030 (Snehalata, 2009). Metformin (1,1-dimethylbiguanide) (Fig 1) acts predominantly by inhibiting hepatic glucose output (Stumvoll et al., 2007; Hundal, 2003) and increased insulin stimulated glucose uptake in skeletal muscles and adipocytes (Bailey, 1996). Its pharmacological mechanisms are different from other class of oral anti-hyperglycemic agents. It is an oral anti-hyperglycemic agent that improves glucose intolerance in patients with type 2 diabetes. Metformin is used in the management of diabetes mellitus since 1957.

It is the most prescribed drug for treatment of type II diabetes (Bailey, 2004). Metformin is drug of choice in management of diabetes because it does not lead to weight gain and is particularly useful in overweight/obese people (American Diabetes, 2009). Metformin has shown to be effective in combination with insulin and other anti-diabetic drug (DeFronzo et al., 1995; Haupt et al., 1991 and Reaven et al., 1992). Rapid and reliable bioanalysis of drug and metabolites concentration in plasma samples is essential for pharmacokinetic study. For pharmaceutical methods, guidelines from the Unites States of Pharmacopeia (USP), International Conference on Harmonization (ICH) and the Food and Drug Administration (FDA) provides a frame work for performing such validations (FDA, 2001). Reversed-phase columns such as C 18 and C 8 have been used in several studies (Sengupta et al., 2009; Zhao et al., 2007; Mistri et al., 2007; Chen et al., 2004 and Marques et al., 2007). Various analytical methods have been reported for the quantification of metformin in human plasma or in pharmaceutical forms, these include ultraviolet (UV) spectrometry (Vesterqvis et al., 1998), Raman spectrometry (Ridente et al., 1999) and HPLC-UV, (Arayne et al., 2013 and Kumar et al., 2014). More sensitive LC–MS–MS methods have also been used for quantification of metformin in plasma (Chen, 2004; Chen et al., 2011 and Marques et al., 2007). Method validation is the process of proving that an analytical method is accepted for its intended
purposes. In order to investigate pharmacokinetic properties of the metformin, a simple, sensitive and specific method for analysis of metformin in biological fluid such as plasma is required. The present study describes bioanalytical method for the determination of metformin in rat plasma after oral administration. This method has been successfully applied in the pharmacokinetic studies of metformin in Sprague Dawley rats.

**Experimental**

**Chemicals and Materials**

Gift sample of pharmaceutical grade Metformin and Phenformin (Internal standard, IS) were provided by Zydus Cadila Limited, (Sikkim, India). HPLC grade acetonitrile and methanol were purchased from Sigma Aldrich Chemicals Pvt. Ltd. (Mumbai, India). Sodium acetate AR, glacial acetic acid AR, and ammonia solution (25%) were purchased from E Merck Pvt. Ltd (Mumbai, India). Oasis HLB 1 c.c., 30 mg solid phase extraction cartridges were procured from Waters. MilliQ ultrapure water was obtained from a Milli-Q plus water purification system. Heparin sodium injection I.P. (1000 IU/mL) was purchased from Cadila Limited (Mumbai, India). Oasis HLB 1 c.c., 30 mg solid phase extraction cartridges were procured from Waters. Ultra pure water was obtained from a Milli-Q PLUS water purification system. Heparin sodium injection I.P. (1000 IU/mL) was purchased from Biologicals E. Limited (Hyderabad, India). Animal study was carried out as per the approval and guidelines of the Institutional Animal Ethical Committee (IAEC) of King George’s Medical University UP Lucknow India.

**Instrumentation and chromatographic conditions**

The method was developed using a Waters HPLC system (Milford USA) consisting of a binary pump (model 515), auto sampler (model 717) and photo diode array (PDA) detector. The optimum chromatographic conditions were achieved using Thermo AccuCore Hillyc (150 × 4.6 mm, 5 µm) column with a mobile phase consisting 20 mM sodium acetate buffer and acetonitrile (45:55, v/v with pH-4); at a flow rate of 0.7 ml min⁻¹. Mobile phase was filtered and degassed by passing through 0.45 micron nylon filter (Millipore, Bedford, MA) under vacuum followed by sonication for 15 min. Detection was carried out with PDA at 236 nm wave length. Total run time was 6 min and volume of injection was 50 µl. To minimize carryover, the sample loop was washed between injections with 50% methanol in deionized water. Before each batch of sample, the analytical column was equilibrated with Solution A for at least 1.5 h at a flow rate of 1 ml/min and with the mobile phase for 30-40 min. Analysis was performed at ambient temperature. The HPLC data collection and analysis were performed using breeze - version 3.1 software (Waters).

**Preparation of Stock and standard Solution**

Primary stock solution of metformin and internal standard (IS) for preparation of calibration curve and quality control (QC) samples were prepared from separate weighing. The primary stock solution of analyte and IS was prepared in methanol/H₂O (50:50, v/v) (normal concentrations 1.0 mg/ml).

**Table 1. Intra-day and inter-day assay precision and accuracy for Metformin**

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Accuracy (%Bias)</th>
<th>Precision (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-day</td>
<td>Inter-day</td>
</tr>
<tr>
<td>0.062</td>
<td>3.35</td>
<td>3.75</td>
</tr>
<tr>
<td>0.25</td>
<td>0.27</td>
<td>-0.52</td>
</tr>
<tr>
<td>2.0</td>
<td>0.06</td>
<td>0.43</td>
</tr>
</tbody>
</table>

**Table 2. Stability of metformin in rat plasma**

<table>
<thead>
<tr>
<th>Nominal Conc. (µg/ml)</th>
<th>Stability</th>
<th>Mean</th>
<th>S.D.</th>
<th>Precision (%RSD)</th>
<th>Accuracy (%bias)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.062</td>
<td>Auto sampler stability</td>
<td>0.0628</td>
<td>0.011</td>
<td>0.41</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>Bench-top stability</td>
<td>0.0621</td>
<td>0.013</td>
<td>1.75</td>
<td>-6.82</td>
</tr>
<tr>
<td></td>
<td>Freeze-Thaw stability</td>
<td>0.0625</td>
<td>0.019</td>
<td>1.71</td>
<td>-0.50</td>
</tr>
<tr>
<td></td>
<td>Long-term stability</td>
<td>0.0625</td>
<td>0.12</td>
<td>2.03</td>
<td>4.5</td>
</tr>
<tr>
<td>2.0</td>
<td>Auto sampler stability</td>
<td>2.03</td>
<td>0.05</td>
<td>2.36</td>
<td>-0.77</td>
</tr>
<tr>
<td></td>
<td>Bench-top stability</td>
<td>2.08</td>
<td>0.08</td>
<td>2.79</td>
<td>-5.44</td>
</tr>
<tr>
<td></td>
<td>Freeze-Thaw stability</td>
<td>2.05</td>
<td>0.13</td>
<td>3.37</td>
<td>-0.6</td>
</tr>
<tr>
<td></td>
<td>Long-term stability</td>
<td>1.99</td>
<td>0.11</td>
<td>1.70</td>
<td>3.49</td>
</tr>
</tbody>
</table>

**Table 3. Pharmacokinetic parameters of metformin in SD rats following oral administration 100 mg/Kg**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Estimates</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC₀→∞ (min*µg/mL)</td>
<td>1970 ± 105.51</td>
</tr>
<tr>
<td>t₁/₂ (min.)</td>
<td>384 ± 34.61</td>
</tr>
<tr>
<td>C₀ (µg/mL)</td>
<td>13.79 ± 3.15</td>
</tr>
<tr>
<td>CL (mL/min.)</td>
<td>31.1 ± 7.1</td>
</tr>
<tr>
<td>Tamax (min.)</td>
<td>190 ± 49.32</td>
</tr>
<tr>
<td>MRT (min.)</td>
<td>454± 38.6</td>
</tr>
</tbody>
</table>

Abbreviation: C₀: concentration at time zero, AUC: area under the curve from 0 to ∞ hr, CL: clearance, t₁/₂: terminal half life, MRT: mean residence time. Values are represented as mean ± SD Calibration standard (CS) sample was prepared by spiking respective stock solutions in blank rat plasma pool at concentrations of 0.062, 0.125, 0.25, 0.5, 1.0, and 2 µg/mL. Metformin stock solution for QC was prepared separately. QC samples at three different concentrations (0.062, 0.25 and 2 µg/mL as LLOQ, medium and high, respectively) was prepared separately in ve replicates, independent of the calibration standards. Calibration curve was plotted as concentration of drugs versus peak area response. QC samples were prepared from different matrix pools on each day of analysis. Prepared plasma samples were stored at -15 C or below and all prepared stock solutions were stored at 4 C until analysis.
Sample Preparation

A solid phase extraction (SPE) method was followed for extraction of metformin in rat plasma. CS, QC and plasma samples were extracted using SPE method. An aliquot of sample mixture (200 µl) of blank rat plasma was mixed with 10 µl of IS (5 µg/ ml). The Oasis HLB SPE cartridge (1 ml, 30 mg) was preconditioned with 1.0 ml methanol followed by 1.0 ml of 0.5% aqueous ammonia solution. Sample mixture and 200 µl of 0.2% aqueous ammonia solution were loaded to the SPE cartridge and cartridge was washed with 2.0 mL of 5 % aqueous methanol. Finally metformin was eluted from the SPE cartridge with 2.0 mL of methanol. The eluate was collected and evaporated to dryness under vacuum in a speedvac concentrator (Savant Instrument, Farmingdale, USA). The residue was reconstituted in 200 µl of mobile phase and 50 µl was injected into the HPLC.

Recovery

The recovery of metformin and IS through SPE procedure, was determined by comparing the responses of the analytes extracted from replicate QC samples (n = 5) with the response of analytes from post-extracted plasma samples at equivalent concentration. Recoveries of metformin were determined at QC low, QC medium and QC high concentrations, viz., 0.062, 0.25 and 2 µg/ml, whereas the recovery of the IS was determined at a single concentration of 5 µg/ml.

Validation procedure

All the validation steps were conducted in accordance to the FDA guide line (FDA 2001). The method was validated to demonstrate the specificity, selectivity linearity, matrix effect, accuracy, limit of quantification, limit of detection, robustness precision and stability. The spec city of the method was evaluated by analyzing blank plasma samples collected from six different rats to investigate the potential interferences in the liquid chromatographic peak region for the analyte and IS using the proposed extraction procedure and chromatographic conditions. Linearity was tested at six concentration levels covering a range of 0.062 – 2.0 µg / ml. The calibration curve was obtained by plotting the peak area ratio (peak area analyte/peak area IS) versus concentration. The results were subjected to a linear regression analysis using weighing factor (1/x²). The acceptance criteria for each back-calculated standard concentration were ± 15% deviation from the nominal value except at the lower limit of quantitation (LLOQ), which was set at ± 20%. The LLOQ of the validation was assessed as the lowest concentration on the calibration curve that could be quantitatively determined with acceptable precision and accuracy within ± 20%. The LOQ was established based on six replicates on five consecutive days. The matrix effect was evaluated in rat plasma by comparing the corresponding peak areas of the post extraction spiked samples to those of standard solutions evaporated directly and reconstituted in mobile phase. Experiments were performed at three QC levels. If the ratio was less than 85% or more than 115%, the matrix effect was regarded significant. Batches consisting of five calibration standards of each concentration and QC samples were analyzed on five days to complete the method validation. In each batch, QC samples at 0.062, 0.25 and 2 µg/ml were assayed in sets of five replicates to evaluate the intra- and inter- day precision and accuracy. The criteria for acceptability of data included accuracy (% bias) and precision which must be within ± 15% of the nominal value and ± 20% at the LLOQ. The accuracy was expressed as % bias:

\[
\% \text{Bias} = \frac{\text{observed conc.} - \text{nominal conc.}}{\text{nominal conc.}} \times 100
\]

All stability studies were carried out at 0.062 and 2.0 µg/ml in five replicates. The freeze-thaw stability was found out after three freeze-thaw cycles (room temperature to -70 ± 10 °C). Post extraction auto-sampler stability of metformin was examined at 4 °C for 72 h and bench-top stability of metformin in rat plasma was evaluated at ambient temperature (25 ± 5 °C) for 24 h. The long-term freezer stability was determined at -80 ± 10 °C over 30 days.

![Fig. 1. Structure of Metformin (analyte) and phenformin (IS)](image1)

Fig. 1. Structure of Metformin (analyte) and phenformin (IS)

![Fig. 2. PDA spectra of mobile phase base line and metformin peak](image2)

Fig. 2. PDA spectra of mobile phase base line and metformin peak
Dilution Integrity

Dilution of biological matrix is required if some study sample concentrations are expected to be higher than the upper limit of quantitation (2.0 \( \mu \text{g/ml} \)). A dilution integrity experiment was performed by seven times dilution of plasma samples containing 14 \( \mu \text{g/ml} \) with blank plasma to obtain sample containing 2.0 \( \mu \text{g/ml} \) (QC high) of metformin. Assay precision and accuracy were determined in a similar as described above.

Application of the method

In-vivo pharmacokinetics study of metformin was performed in male \textit{Sprague Dawley} rats (n=5, weight range 180.0 \( \pm \) 20 gm) to reveal the applicability of developed and validated bioanalytical method (Du, 2015). The metformin was administered orally at a dose of 100 mg/kg in 0.25 % carboxy methyl cellulose suspension (CMC). Blood samples were collected from the retro orbital plexus from rat under mild anesthesia into micro-centrifuge tubes containing heparin (20 IU/ml) as anti-coagulant at 0.25, 0.5, 0.75, 1.0, 2.0, 3.0, 4.0, 6.0, 7.0, 8.0, 10.0, 12.0, 15 and 24 h post dosing. Plasma was harvested by centrifuging the blood samples at 2000 \( \times \) g for 5 min and stored at -80 \( \pm \) 10 °C until analysis. Rat plasma samples (200 µl) were spiked with IS, processed as described above and data was accepted based on performance of QCs samples prepared using rat blank plasma (five QC each at four concentration levels). The criteria for acceptance of analytical run encompassed the following: (i) not more than 33% of QC samples were greater than \( \pm \)15% of the nominal concentration; (ii) not less than 50% at each QC concentration level must meet the acceptance criteria (Bhatta \textit{et al}., 2011; Sultana \textit{et al}., 2013). Plasma concentration-time profile of metformin was analyzed by non-compartmental method using software WinNonlin Version 5.1 (Pharsight Corporation, Mountain view, USA).

RESULT AND DISCUSSION

This method has significance in terms of sensitivity, selectivity and recovery using Accuacore Hillic column. HPLC chromatogram has the better resolution. The established LLOQ of 0.062 \( \mu \text{g/ml} \) was sufficiently low in comparison to published literature. The solid phase extraction method gave consistent and reproducible recovery and minimized the matrix effect. Short run time and less organic solvent involvement in mobile phase were used by this method that’s why method is better than others concerning to time and cost.

HPLC method development

Process of method development began with the optimization of chromatographic condition including mobile phase composition and column type. Analysis of metformin is a challenge owing to its high polarity and small molecular size with high polarity, which lead to poor retention on reversed-phase liquid chromatographic columns. The chromatographic conditions were optimized with respect to speci city, resolution, and time of analysis. The development activity was started with C\textsubscript{18} stationary phase of various manufacturers such
as Zorbax, ODS (250 × 4.6 mm, 5 µm), Spherisorb ODS (250 × 4.6 mm, 5 µm) Symmetry shield C18 (250 mm × 4.6 mm, 5.0 µ), Phenomenex Luna C18 (250 × 4.6 mm, 5 µm), Spheri-5, CYANO column (30 × 4.6 mm, 5 µm) and Thermo Accuacore Hillic (150 × 4.6 mm, 5 µm) column. The last two columns were found to be suitable. However, Thermo Accuacore Hillic (150 × 4.6 mm, 5 µm) column was selected owing to increase optimum retention, good resolution and minimum elution. The stationary phase was not only the parameter which could give better resolution. Mobile phase, pH and organic modifies also played very important role which leads the best separation. Different mobile phases containing acetonitrile, methanol, water and buffer were examined. Initially the methanol was used as an organic modifier which gives poor baseline with baseline drift. Hence the response for the metformin was reduced. To improve the resolution and response, acetonitrile was tried as an organic modifier, which improved baseline and response for metformin.

Effects of pH (3–7) and ionic strength (5–50 mM) were investigated using phosphate and acetate buffer. It was found that at higher and lower pH the tailing of the metformin peak was more and also the resolution of analyte was poor. The effect of buffer concentration on the retention of metformin was also studied. The mobile phase containing acetonitrile: sodium acetate buffer 20 mM (55: 45 v/v, pH 4), was selected as optimal for obtaining well-resolved peaks with acceptable system suitability parameters. Flow rates from 0.5 to 1 ml min⁻¹ were tested. Flow rates less than 0.5 ml/min led to an increase in resolution, peak broadening and the time of analysis. High flow rates lead to a remarkable increase in column pressure and decrease in resolution. It was found that 0.7 ml min⁻¹ was optimal as it compromised between resolution and run time. Effect of the wavelength on the response factor was observed over wavelength range 200–300 nm. The detection wavelength, 236 nm was found optimal due to high absorbitivity at this wavelength for metformin (Fig-2). Complete separation was achieved in < 6.0 min at ambient temperature (Fig. 3). The average retention times ± relative standard deviation (% RSD) % for metformin was found to be 4.4 ± 0.10 (n = 10).

Sample extraction and recovery

The blank plasma was used as a surrogate matrix organic solvent precipitation using acidi ed acetonitrile or methanol and DMSO in methanol resulted in non-reproducible recoveries and interferences from the sample matrix with the chromatography of the analytes (data not shown). Liquid–liquid extraction was not a feasible option due to hydrophilicity of drug. Various researchers agree that the main problem of the metformin analysis from biological samples due to its high polarity. Which makes it difficult to extract the drug directly from biological fluids by organic solvents. Subsequently SPE method has been developed to overcome the polarity of metformin, SPE method for extraction was investigated using Oasis HLB, CN and C18 cartridges for optimizing the extraction procedure. Among these, Oasis HLB cartridges (1 cm³, 30 mg) with several conditioning, washing and elution reagents gave consistent results in terms of recovery of metformin and IS and also gave cleaner plasma sample extract. The use of 0.5% v/v aqueous ammonia solution during the washing and 0.2 % in loading step provided sufficient interaction of Metformin with the SPE bed. This resulted in considerable improvement in recovery of metformin. The absolute mean recovery of Metformin and IS were 73.09% and 65.35% respectively

Validation procedures

Selectivity and specicity

Six lots of blank rat plasma were analyzed for the evaluation of selectivity and specicity. These samples did not show any signi cant interfering peaks at the retention times of either metformin the IS (Fig. 3).

Matrix effect

The adverse consequences of matrix effects on results of quantitative HPLC analyses have been fully recognized and assessment of matrix effects is becoming an integral part of method development and validation. The matrix effect for metformin at 0.062, 0.25 and 2.0 µg/ ml concentration levels in rat plasma was < ± 3% (1.18 - 2.82). Thus no significant matrix effect was observed.

Calibration curves

The peak area ratio of analyte to IS was linear over a concentration range of 0.062 -2.0 µg/ml for metformin. The calibration curve had a reliable reproducibility over concentration range 0.062, 0.125, 0.25, 0.5, 1, 2.0 µg/ml. The average correlation coefficient (r) was found to be 0.997 ± 0.0018. The lowest concentration with RSD < ± 20% was taken as the LLOQ and this was found to be 0.062µg/ml.

Accuracy and precision

Intra- and inter-assay precision was evaluated from the relative standard deviations (% RSD) of the quality control samples (LLQC, MQC, and HQC). The intra-day assay precision and intra-assay accuracy of metformin were within the limits and ranges from 2.49% to 5.64% and 0.27% to 3.32% respectively (Table 1). The inter-day assay precision and accuracy ranged from 2.17% to 5.59% and -0.51% to 3.72% respectively. Both intra and inter-day precision and accuracy were determined to be within accepted variable limits.

Stability

The predicted concentrations for metformin at 0.062 and 2.0 µg /ml samples deviated within the nominal concentrations in a battery of stability tests, viz., auto sampler stability (24 h), bench-top stability (6 h), repeated three freeze-thaw cycles and at -70 ± 10 ºC for 30 days (Table 2). The results were found to be within assay variability limits during the validation process.

Dilution integrity

Dilution integrity experiment carried out at four replicates by seven times dilution with blank plasma and assay precision and
accuracy were determined in a similar manner as described above. The % accuracy of diluted QCs was in the range of 104–108, while precision (% R.S.D.) for diluted QCs was 0.87. These results indicated that dilution of samples up to seven fold with blank plasma does not compromise the assay.

Application of the method

For pharmacokinetic analysis, the rat plasma samples obtained after oral administration of metformin were analyzed by the newly developed and validated method along with the QCs samples. The specificity and sensitivity of the assay were found to be sufficient for accurately characterizing the pharmacokinetics of metformin in rats. The plasma concentration-time pro le and pharmacokinetic estimates of metformin is given in Fig. 4 and Table 3 respectively. Concentration-time curve was smooth enough to estimate pharmacokinetic parameters and the elimination phase was well fitted to first order non compartment pharmacokinetic model. Maximal Metformin plasma levels after oral administration of 100 mg/kg metformin was 13.79 ± 3.15 µg/ml. The Mean residence time (454± 38.6 min) was found. Parameters t1/2 (384 ± 34.61 min) and AUC (1970 ± 105 min*µg/ml) do not indicate rapid elimination of metformin.

Conclusion

In summary, a sensitive, rapid and specific HPLC assay of metformin using Accuacore Hichic column was developed and validated. A good linearity was obtained over the concentration range of 0.062-2.0 µg/ml. Good recovery was obtained using SPE method. Moreover, the assay demonstrate a high sensitivity with LLOQ 0.062 µg/ml using 200 µL volume of plasma sample and simple solid phase extraction procedure. The method is accurate, precise, reproducible and was applied successfully in pharmacokinetic studies of metformin in the SD rats.

Acknowledgements

The authors are thankful to Vice chancellor, King George’s Medical University, Uttar Pradesh Lucknow, India, for his constant encouragement and support. We also acknowledge Indian Council of Medical Research (ICMR) for providing research fellowships to one of the authors.

REFERENCES


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