Rapid RP-HPLC Method for Simultaneous Estimation of Metformin, Pioglitazone, and Glimepiride in Human Plasma

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An isocratic reversed-phase high-performance liquid chromatography (RP-HPLC) method has been developed for rapid and simultaneous separation and estimation of 3 antidiabetic drugs, namely, metformin, pioglitazone, and glimepiride, in human plasma within 3 min. Separation was carried out on a MAGELLEN 5U C18 (5 μm, 150 mm × 4.60 mm) using a mobile phase of MeOH-0.025 M KH2PO4 adjusted to pH 3.20 using ortho-phosphoric acid (85:15, v/v) at ambient temperature. The flow rate was 1 mL/min, and the maximum absorption was measured at 235 nm. The retention time of metformin, pioglitazone, and glimepiride was noted to be 1.24, 2.32, and 2.77 min, respectively, indicating a very short analysis time compared to that of other reported methods. Also, limits of detection were reported to be 0.05, 0.26, and 0.10 μg/mL for metformin, pioglitazone, and glimepiride, respectively, showing a high degree of method sensitivity. The method was then validated according to the FDA guidelines for the determination of the three drugs clinically in human plasma, in particular, regarding pharmacokinetic and bio-equivalence simulation studies.

Keywords: RP-HPLC, metformin, pioglitazone, glimepiride, human plasma

1. Introduction

Oral antidiabetic drugs are widely used for treatment of diabetes mellitus type II. It is the most common diabetes disease caused by fatty acids and myocells resistance to insulin. Its treatments include insulin secretagogue agents that increase the amount of insulin secreted from the pancreas, insulin sensitizer agents that increase the sensitivity of target organs to insulin, or agents that decrease the rate of gastrointestinal tract absorption of glucose [1].

Chemically, metformin (MET) is 1,1-dimethyl biguanide (Figure 1). It decreases the gluconeogenesis process and increases the glucose uptake by muscles and fat cells. It is the cornerstone for the treatment of diabetes mellitus type II, where it is used alone or in combination with other antidiabetic classes like sulfonylureas, alphaglycosidase inhibitors, or insulin [2].

Our literature survey verified that determination of metformin has been carried out in tablets by high-performance liquid chromatography (HPLC) [3–6], in human plasma using ion-pair HPLC [7], or through using capillary electrophoresis [8, 9].

Pioglitazone (PIO) is a thiazolidinedione antidiabetic agent and chemically is (RS)-5-(2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamide)ethyl)[phenyl]sulfonyl]-3-(trans-4-methylcyclohexyl)urea (Figure 1). Many chromatographic [23–25] and spectrophotometric methods [26–30] were also reported for the determination of glimepiride, either alone or in combination with other antidiabetic drugs.

To the best of our knowledge and comprehensive survey, metformin, pioglitazone, and glimepiride mixture in dosage form was determined by reversed-phase HPLC (RP-HPLC) [3, 31–33] but was never determined before by chromatographic techniques in biological samples despite their synergistic action. As such, the present work introduces a simple, very rapid, reproducible, and sensitive chromatographic method for the determination of the three drugs in human plasma. Also, this method has another advantage to protect analytical scientists and chemists from the exposure to volatile agent due to its consistent secretagogue action [22]. Chemically, it is 1-{[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamide)ethyl][phenyl]sulfonyl]-3-(trans-4-methylcyclohexyl)urea (Figure 1). Many chromatographic [23–25] and spectrophotometric methods [26–30] were also reported for the determination of glimepiride, either alone or in combination with other antidiabetic drugs.

1.2. Metformin

Metformin (MET) is a potent antidiabetic oral agent due to its consistent secretagogue action [22]. Metformin is 1,1-dimethyl biguanide which is a non-insulin secretagogue drug used primarily for treating type 2 diabetes mellitus [1]. Metformin is a biguanide oral drug that works by decreasing glucose production in the liver and increasing glucose uptake in muscle and fat cells. Metformin is generally well tolerated and does not cause insulin-like adverse effects, making it a preferred initial treatment for type 2 diabetes mellitus [2].

1.3. Pioglitazone

Pioglitazone (PIO) is a thiazolidinedione antidiabetic agent and chemically is (RS)-5-(2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamide)ethyl)[phenyl]sulfonyl]-3-(trans-4-methylcyclohexyl)urea (Figure 1). Many chromatographic [23–25] and spectrophotometric methods [26–30] were also reported for the determination of glimepiride, either alone or in combination with other antidiabetic drugs.

1.4. Glimepiride

Glimepiride (GLM) is a potent third generation sulfonylurea derivative and it is widely used in the treatment of non-insulin-dependent diabetes mellitus type II as an oral hypoglycemic agent due to its consistent secretagogue action [22]. Chemically, it is 1-{[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamide)ethyl][phenyl]sulfonyl]-3-(trans-4-methylcyclohexyl)urea (Figure 1). Many chromatographic [23–25] and spectrophotometric methods [26–30] were also reported for the determination of glimepiride, either alone or in combination with other antidiabetic drugs.

To the best of our knowledge and comprehensive survey, metformin, pioglitazone, and glimepiride mixture in dosage form was determined by reversed-phase HPLC (RP-HPLC) [3, 31–33] but was never determined before by chromatographic techniques in biological samples despite their synergistic action. As such, the present work introduces a simple, very rapid, reproducible, and sensitive chromatographic method for the determination of the three drugs in human plasma. Also, this method has another advantage to protect analytical scientists and chemists from the exposure to volatile

Figure 1. Chemical structures of metformin (MET), pioglitazone (PIO), and glimepiride (GLM)
and corrosive organic solvents during experimentation using an environmentally benign mobile phase.

2. Experimental

2.1. Apparatus.
- Agilent® 1200 HPLC instrument (Germany) with a MAGELLEN 5U C18 5 μm (150 mm × 4.60 mm), a diode-array-detector (DAD) absorbance detector, and Agilent quaternary pumps, and connected to a PC computer loaded with Agilent 1200 HPLC.
- Labomed® Spectro UV-vis Double Beam (UVD-2950) spectrophotometer with matched 1-cm quartz cells and connected to windows-compatible computer using UV Win S Software v6.
- Hanna® HI 8314 membrane pH meter (Romania) for pH adjustment.

2.2. Materials and Reagents.
- All solvents and reagents were of HPLC-analytical grade (methanol, potassium dihydrogen phosphate, and ortho-phosphoric acid were provided by Fisher Scientific, England).
- Metformin, pioglitazone, and glimepiride. Standard solutions of 200 μg/mL were prepared by dissolving 0.01 mg of each pure drug in 50 mL of the mobile phase.
- Mobile phase was a freshly prepared binary mixture of MeOH-0.025 M potassium dihydrogen phosphate adjusted to pH 3.20 using ortho-phosphoric acid (85:15, v/v), filtered and degassed using a 0.45-μm membrane filter.
- The human plasma was kindly provided by Zagazig University Hospital and was tested to be drug and disease-free. The plasma was kept frozen before use and was then stored either at −4 °C between uses or at −20 °C for freeze-thaw cycle stability studies.

2.3. Procedures

2.3.1. Preparation of Standard Calibration Curves. Appropriate mixed dilutions of metformin, pioglitazone, and glimepiride standard stock solutions were done in 10-mL volumetric flasks to get final concentrations of 2.50, 5, 10, 1250, 25, 50, and 100 μg/mL for all drugs. A 10 μL of each mixture was then injected into the column, and the chromatogram was obtained at 235 nm. A graph was plotted as concentration of drugs against response (peak area). Regarding validated quality control (QC) samples, concentrations of 25, 50, and 100 μg/mL were selected as low (LQC), medium (MQC), and high (HQC) levels, respectively.

2.3.2. Human Plasma Samples Procedure. Calibration curve and validated QC samples at concentrations of 5, 10, and 15 μg/mL in plasma were prepared. Aliquots of 200 μL plasma samples and different drug mixture volumes ranging from 100 up to 200 μL were added into 10-mL centrifuge tubes and vortexed for 1 min. The mixture was then precipitated with methanol (total volume: 2 mL). After vortexing for 1 min, the samples were centrifuged at 5000 rpm for 15 min. Aliquots of 10 μL of the supernatant were filtered through 0.45-μm PTFE syring filters (Membrane Solutions, USA) and injected into the HPLC system for analysis.

3. Results and Discussion

3.1. Optimization of Chromatographic Conditions. All chromatographic conditions are illustrated in Table 1. Spectroscopic analysis of the three drugs in the range of 200–400 nm showed that metformin, pioglitazone, and glimepiride have UV absorbance maxima (λmax) at 237, 227, and 229 nm, respectively, as depicted in Figure 2. Therefore, the chromatographic detection was performed at 235 nm as the appropriate wavelength using a DAD detector. The method was performed on a MAGELLEN 5U C18 5 μm (150 mm × 4.60 mm).

Table 1. Chromatographic conditions for the proposed method

| Parameters          | Conditions          |
|---------------------|---------------------|
| Column              | MAGELLEN C18 (5 μm, 150 mm × 4.60 mm) |
| Mobile phase        | Isocratic binary mobile phase of MeOH-0.025 M KH2PO4 adjusted to pH 3.20 using ortho-phosphoric acid (85:15, v/v), filtered and degassed using a 0.45-μm membrane filter |
| UV detection (nm)   | 235                  |
| Flow rate (mL/min)  | 1                    |
| Injected volume (μL) | 10                   |
| Pressure (psig)     | 98                   |
| Temperature         | Ambient              |

Furthermore, under several trials of mobile phase optimization regarding its composition ratio and pH, it was observed that the optimized mobile phase was determined as a mixture of MeOH and 0.025 M potassium dihydrogen phosphate adjusted to pH 3.20 using ortho-phosphoric acid (85:15, v/v) at a flow rate of 1 mL/min. Under these conditions, metformin, pioglitazone, and glimepiride can be separated and eluted at 1.24, 2.32, and 2.77 min, respectively as illustrated in Figure 3. Also, the mixture determination in plasma didn't show the matrix interference effect, as the human plasma was eluted separately at 1.45 min in correspondence with the migration times of the three drugs (Figure 4A and B). However, in all cases, the optimum mobile phase showed symmetrical peaks (0.69 < T < 0.88), capacity factor (k < 10), resolution > 2, and theoretical plates > 2000. Table 2 shows all system suitability parameters of the proposed RP-HPLC method for simultaneous determination of the three antidiabetic drugs in both pure and plasma samples.

3.2. Method Validation. The method was validated according to the Food and Drug Administration guidelines for bioanalytical methods validation [34–36].

3.2.1. Linearity. Five different concentrations of the drug mixture were specified for linearity studies. The calibration curves obtained by plotting peak area against concentration showed linearity in the concentration range of 2.50–100 μg/mL for all drugs (Table 3). Linear regression equations for metformin, pioglitazone, and glimepiride were found to be $y = 61.08x + 99.75$, $y = 12.22x + 24.98$, and $y = 29.62 + 41.84$, respectively, and the regression coefficient values ($r$) were 0.999 for the three drugs, indicating a high degree of linearity (Figure 5).

3.2.2. Accuracy and Precision. The accuracy of the method was determined by investigating the recoveries of metformin, pioglitazone, and glimepiride at concentration levels covering the specified range (3 replicates of each concentration). From the amount of the drug estimated, the percentage recovery was

Figure 2. Overlaid spectra of 1 μg/mL metformin (MET), pioglitazone (PIO), and glimepiride (GLM) at maximum wavelengths of 237, 227, and 229 nm, respectively.
calculated, and the results shown in Table 1 indicate excellent recoveries for all drugs.

The precision of the method was evaluated according to intra-day and inter-day precision using validated QC samples.

Figure 3. HPLC chromatogram of a 2.5 μg/mL authentic mixture of metformin (MET), pioglitazone (PIO), and glimepiride (GLM) using MAGELLEN C18 (5 μm, 150 mm × 4.6 mm) column and a mobile phase of MeOH–0.025 M KH2PO4 adjusted to pH 3.20 using ortho-phosphoric acid (85:15, v/v). Other chromatographic conditions are stated in Table 1.

Figure 4. HPLC Chromatogram of (A) blank plasma sample and (B) 2.5 μg/mL authentic mixture of metformin (MET), pioglitazone (PIO), and glimepiride (GLM) in human plasma under the same conditions of Figure 3.
Table 2. System suitability parameter comparison for metformin (MET), pioglitazone (PIO), and glimepiride (GLM) in both pure and plasma samples

| Parameters                  | Pure sample | Plasma sample | Reference values [36] |
|-----------------------------|-------------|---------------|-----------------------|
| MET                         | PIO         | GLM           |                       |
| Retention time, \( t_r \)   | 1.24        | 2.32          | 2.77                  | 1.25                     | 2.35          | 2.79         |               |
| Capacity factor, \( k' \)   | 0.79        | 1.13          |                       | 0.81                     | 1.14          |               | Accepted \( k' \) value (1–10) |
| Peak asymmetry (tailing factor, \( T \)) | 0.70  | 0.80          | 0.87                  | 0.69                     | 0.73          | 0.88         | Accepted \( T \) value ≤ 2 |
| Theoretical plates, \( N \) | 2865        | 4085          | 4588                  | 2101                     | 4270          | 4100         | Accepted \( N \) value > 2000 |
| Resolution, \( R_s \)       | –           | 8.93          | 2.96                  | –                        | 8.77          | 2.91         |               |
| Selectivity (separation factor, \( \alpha \)) | –           | –             | 1.43                  | –                        | –             | 1.41         |               |

Table 3. Results of analysis for the proposed method

| Parameters                  | MET          | PIO          | GLM          |
|-----------------------------|--------------|--------------|--------------|
| Taken (µg/mL)               | Found (µg/mL) | Recovery (%) | Accuracy (%) | Taken (µg/mL) | Found (µg/mL) | Recovery (%) | Accuracy (%) | Taken (µg/mL) | Found (µg/mL) | Recovery (%) | Accuracy (%) |
| Mean                        | 99.46        | 0.53         | 1.09         | 100.51       | 1.51         | 2.50         | 2.46         | 98.79         | 1.20          |
| ±SD                         | 1.15         |              | 1.15         | 1.08         |              | 25.0         | 26.5         | 98.60         | 1.39          |
| ±RSD                        | 1.15         |              | 0.51         | 0.48         |              | 50.0         | 50.4         | 100.82        | 0.82          |
| ±SE                         | 1.32         |              |              | 1.19         |              | 100.23       | 0.23         | 99.87         | –0.77         |
| Variance                    | 1.32         |              |              |              |              |              |              |              |               |
| LOD (µg/mL)                 | 0.05         | 0.26         |              | 0.10         |              |              |              |               |
| LOQ (µg/mL)                 | 0.18         | 0.89         |              | 0.35         |              |              |              |               |

Figure 5. Calibration curves for authentic mixture of metformin (MET), pioglitazone (PIO), and glimepiride (GLM) using the proposed HPLC method

at concentrations of 25, 50, and 100 µg/mL. Intra-day precision was evaluated in terms of both standard deviation (SD) and coefficient of variation (CV%), regarding 3 replicate determinations using the same solution containing pure drugs at the first day of analysis. The SD and CV% values (varied from 0.07 to 1.16) revealed the high precision of the method. For inter-day reproducibility, the day-to-day SD and CV% values were also in the acceptable range of 0.04–0.93. These results show that the proposed method has an adequate precision in simultaneous determination of the three drugs.

3.2.3. Selectivity and Specificity. The selectivity of the method was checked by injecting the solutions of metformin, pioglitazone, and glimepiride into the column separately, where 3 sharp peaks were obtained at retention times of 1.24, 2.32, and 2.77 min, respectively, and these peaks were not obtained for the blank solution. Also, the specificity studies revealed that the presence of human plasma didn’t show any kind of interference with the sharp and well-resolved peaks of the three drugs (Figure 4).

3.2.4. Robustness. The robustness of the methods was evaluated by making deliberate subtle changes (+0.05) in the flow rate, pH of mobile phase, and mobile phase composition ratio, keeping the other chromatographic conditions constant. The changes effect was studied on the basis of percent recovery and standard deviation of all drugs. Table 4 shows that the changes had negligible influences on the results as revealed by small SD values (±1.69).

3.2.5. Limits of Detection and Limits of Quantification. For determining the limits of detection and quantitation, the method based on the signal-to-noise ratio (3:1 for LOD & 10:1 for LOQ) was adopted. Limits of detection were reported to be 0.05, 0.26, and 0.10 µg/mL, while limits of quantification were calculated to be 0.18, 0.894 and 0.89, and 0.35 µg/mL for metformin, pioglitazone, and glimepiride, respectively (Table 3). These results show that the proposed method is highly sensitive and applicable for pharmacokinetic and bioequivalence studies, where detection of small concentrations in plasma is required.

Table 4. Results of the robustness for the determination of 12.5 µg/mL metformin (MET), pioglitazone (PIO), and glimepiride (GLM)

| Parameters                  | MET          | PIO          | GLM          |
|-----------------------------|--------------|--------------|--------------|
| Mean recovery ± SD          | CV (%)       | Accuracy (%) | CV (%)       | Accuracy (%) | CV (%)       | Accuracy (%) |
| Flow rate 0.95 mL (+0.05)   | 99.51 ± 1.23 | 1.51         | –0.49        |              |              |              |
| Flow rate 1.05 mL (+0.05)   | 99.49 ± 1.20 | 1.46         | –0.50        |              |              |              |
| Buffer pH 3.15 (+0.05)      | 99.42 ± 1.08 | 1.18         | –0.57        |              |              |              |
| Buffer pH 3.25 (+0.05)      | 99.44 ± 1.11 | 1.25         | –0.55        |              |              |              |
| MeOH–buffer 85:15:5         | 99.39 ± 1.03 | 1.07         | –0.61        |              |              |              |
| MeOH–buffer 84:5:15:5       | 99.36 ± 0.99 | 0.99         | –0.63        |              |              |              |
| Mean recovery ± SD          | CV (%)       | Accuracy (%) | CV (%)       | Accuracy (%) | CV (%)       | Accuracy (%) |
| 100.10 ± 1.55               | 0.08         | 2.40         |              |              |              |              |
| 99.71 ± 0.98                | –0.29        | 0.96         |              |              |              |              |
| 99.57 ± 0.88                | –0.42        | 0.79         |              |              |              |              |
| 99.67 ± 0.94                | –0.33        | 0.89         |              |              |              |              |
| 99.90 ± 1.22                | –0.10        | 1.50         |              |              |              |              |
3.2.6. Analysis of Human Plasma. The proposed method was also applied for determination of metformin, pioglitazone, and glimepiride in human plasma samples by applying protein precipitation procedure. Retention times of metformin, pioglitazone, and glimepiride in plasma sample and the other system suitability parameters were also pretty similar to those of pure one (Table 2).

Also, the plasma chromatogram (Figure 4) confirms the specificity of the method in clinical studies as the plasma peak (eluting at 1.45 min) is not interfering but well separated from the other three peaks of studied drugs. Calibration curves of the spiked plasma were also found to be linear over the clinical range of 5–50 μg/mL for all drugs (Table 5). The mean recoveries for drugs in plasma were in the acceptable range of 80.08–85.10 according to the FDA guidelines. Furthermore, stability studies were conducted by applying plasma freeze–thaw cycles at −20 °C (over three days) using the same validation QC samples in plasma, and the results are summarized in Table 6. The SD and CV% values (varied from 0.21 to 1.92) show that the spiked drug plasma samples are highly stable, indicating the high suitability of the method in pharmacokinetic and bioequivalence studies regarding the three antidiabetic drug mixture in clinical samples.

4. Conclusion

The presented method was developed and validated for rapid simultaneous estimation of metformin, pioglitazone, and glimepiride within 3 min. The results obtained indicate that the proposed method is rapid, accurate, selective, robust, and reproducible. This analytical method can be also adequate and useful for the clinical estimation of metformin, pioglitazone, and glimepiride in human plasma samples according to the FDA guidelines in respect of pharmacokinetic and bioequivalence studies that would be useful in therapeutic drug monitoring.

Compliance with Ethical Standards

Conflict of Interest. The authors declare that there is no conflict of interest in the manuscript.

Ethical Approval. This manuscript does not include any studies on human or animals.

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