Simultaneous Determination and Pharmacokinetic Study of Metformin and Pioglitazone in Dog Plasma by LC–MS-MS

Xueyuan Zhang1*, Ying Peng1, Ping Wan1, Lifang Yin2, Guangji Wang1 and Jianguo Sun1†

1Key Lab of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing, 210009, PR China, and
2School of Pharmacy, China Pharmaceutical University, Nanjing, 210009, PR China

Received 12 July 2012; revised 16 November 2012

A high-performance liquid chromatography–tandem mass spectrometry method was developed for the simultaneous determination of metformin (MET) and pioglitazone (PIO) in dogs. To increase the reliability of the method, moroxydine (IS-1) and rosiglitazone (IS-2) were used as internal standards for MET and PIO, respectively. The analytes were extracted from beagle dog plasma by a one-step protein precipitation. Chromatographic separation was performed on a Phenomenex Synergi POLAR-RP 80A column (250 × 4.6 mm, 4 μm). The total chromatographic run time was 8.0 min, with retention times of 3.0 and 2.9 min for MET and IS-1, respectively, and 6.1 and 4.9 min for PIO and IS-2. The lower limit of quantitation values were 2 and 1 ng/mL for MET and PIO, respectively. The recoveries ranged from 96.4 to 112.8% for MET and 102.1 to 104.5% for PIO.

Introduction

Diabetes is commonly classified into two categories: type 1, insulin-dependent diabetes, and type 2, non-insulin-dependent diabetes. Researchers predict that over the next decade the number of diabetic patients worldwide will exceed 200 million, and most will have type 2 diabetes, which includes risks of the development of complications (1). For many patients with type 2 diabetes, monotherapy with only one antidiabetic agent cannot attain target glycaemic control aims; therefore, combination regiments have become necessary to treat type 2 diabetes.

Metformin hydrochloride (MET), a biguanide antihyperglycemic drug, is used as the first-line oral treatment for type 2 diabetes, which can improve the sensitivity of hepatic and peripheral tissue. Pioglitazone hydrochloride (PIO) is an oral thiazolidinedione antidiabetic agent that has been shown to affect the abnormal glucose and lipid metabolism associated with insulin resistance by enhancing insulin action on peripheral tissues. The effect of lowering blood glucose through the combination of MET and PIO is significantly better than monotherapy. Thus, it is desirable to simultaneously determine the plasma concentrations of MET and PIO, utilizing a sensitive and reliable method.

Validated assays have been reported for each individual drug. For the determination of MET in human plasma, several methods of analysis have been published that use high-performance liquid chromatography (HPLC) with spectrophotometric detection (2), ultraviolet (UV) detection (3–4), and high-performance thin-layer chromatography or thin-layer chromatography (HPTLC/TLC) determination (5). Most of these methods possess low sensitivity and require time-consuming sample preparation. More recent liquid chromatography–tandem mass spectrometry (LC–MS-MS) methods have been reported for the determination of MET, either alone or in combination with other compounds, and have shown high sensitivity (6–11). Bioanalytical methods for the determination of PIO or its metabolites have also been reported, which include HPLC with UV detection (12–13) and LC–MS–MS, with markedly improved sensitivity (14–15).

Due to the differences in pKa values and polarities, it is generally not practical to chromatograph both MET and PIO together when biological samples are involved. Methods for the simultaneous determination of MET and PIO in their combined tablet formulations have also been reported (16–19), including HPLC with UV detection (16), spectrophotometric methods (17) and reversed-phase HPLC methods (18–19). However, these methods are only suitable for the analysis of the drugs in formulations, and are not applicable to pharmacokinetic studies in biological samples because of endogenous interferences and low sensitivity. One report (20) described the simultaneous detection of MET, PIO and glimepiride in human plasma by LC–MS–MS, but in the method, large volumes of plasma (500 μL) were used and liquid–liquid extraction was required. The reported lower limit of quantitation (LLOQ) values of MET and PIO were 10 and 2.5 ng/mL, respectively.

In this present study, an LC–MS–MS method was developed for the simultaneous determination of MET and PIO, employing moroxydine and rosiglitazone as the internal standards (IS). Separation was conducted on a polar embedded Phenomenex Synergi POLAR-RP 80A column (250 × 4.6 mm, 4 μm). The plasma samples were prepared by a single-step protein precipitation with acetonitrile. The observed LLOQ of MET and PIO were 2 and 1 ng/mL, respectively. The method was subsequently validated and successfully applied to a pharmacokinetic study of compound MET and PIO tablets in beagle dogs.

Experimental

Chemicals and reagents

MET hydrochloride, PIO hydrochloride, moroxydine hydrochloride (IS-1) and rosiglitazone hydrochloride (IS-2) reference standards were purchased from the National Institute for
Control of Pharmaceutical and Biological Products (Beijing, PR China). ACTOplus MET XR tablets (PIO and MET extended release, 30/1,000 mg; Takeda Pharmaceutical Company, Japan) were supplied by Dr. Lifang Yin, School of Pharmacy of China Pharmaceutical University.

Ammonium acetate and formic acid were from J&K Scientific. HPLC grade acetonitrile was obtained from Fisher Scientific (St. Louis, MO). Deionized water was generated by passing distilled water through a Quantum EX ultrapure organic cartridge (Millipore S.A. 67120, France).

**LC–MS–MS instrument and conditions**

The analysis was performed on a Finnigan TSQ Quantum Discovery MAXTM LC–MS–MS system equipped with a Finnigan Surveyor LC Pump, an autosampler and a triple-quadrupole mass spectrometer (Thermo Electron Corporation, Palo Alto, CA). The analytical column was a Phenomenex Synergi POLAR-RP 80A (250 x 4.6 mm, 4 µm) and the column temperature was set at 40°C. The mobile phase consisted of acetonitrile–water with 6 mM ammonium acetate and 0.1% formic acid (50:50, v/v) at an isocratic flow rate of 1.0 mL/min. The LC flow was split so that approximately 0.2 mL/min entered the mass spectrometer. The sample injection volume was 10 µL and the run time was 8.0 min. After 1.0 min, the LC eluent was diverted from waste to the mass spectrometer, which was fitted with an electro spray ionization (ESI) source and operated in the positive ion mode, and at 7.0 min, the LC eluent was diverted to the waste again. Data acquisition and processing were performed with the Finnigan Xcalibur 2.0 software (Thermo Electron Corporation).

For quantitation, the mass spectrometer was operated in the selected reaction monitoring (SRM) mode. The spray voltage was 5 kV and the capillary temperature was set at 204°C. The sheath gas and auxiliary gas (N2) pressure were 35 and 10 arb, respectively. Argon was used as the collision gas, with a collision cell gas pressure of 1.5 mtorr (1 torr = 133.3 Pa). The optimized collision energies were 15 eV for MET, 20 eV for IS-1, 28 eV for PIO and 26 eV for IS-2. The monitored ion transitions were m/z 130.0 → 60.1 for MET, m/z 172.0 → 113.0 for IS-1, m/z 356.8 → 133.9 for PIO and m/z 357.9 → 135.0 for IS-2. The scan width for SRM was 0.01 m/z and scan time was 0.2 s. The peak width settings for both Q1 and Q3 were 0.7 m/z.

**Standard solutions**

All concentrations of MET, IS-1, PIO and IS-2 refer to the free bases. Stock solutions of MET and PIO were prepared in water and acetonitrile, respectively. Stock solutions of MET (MET#1, MET#2) and PIO (PIO#1, PIO#2) were prepared separately in duplicate at the concentration of 1 mg/mL, one for calibration curve samples and the other for quality control (QC) samples. The stock solutions of IS-1 and IS-2 at 0.8 and 1 ng/mL were prepared separately in acetonitrile. All of the solutions were stored at 4°C and were determined to be stable for three months.

The mixed IS-1 and IS-2 solution was diluted with acetonitrile to a concentration of 50/50 ng/mL in a 500 mL brown-colored glass bottle. This solution containing the two internal standards (50/50 ng/mL) was used to precipitate the protein for sample preparation.

**Calibration curve and quality control samples**

Mixed working solutions of MET and PIO were serially diluted daily from MET#1 and PIO#1 stock solutions with mixed deionized water and acetonitrile (1:1, v/v). Calibration curves were prepared by spiking 45 µL of blank plasma with 5 µL of corresponding working solutions to produce final concentrations of 1/1, 2/2, 5/5, 10/10, 20/20, 50/50, 100/100, 200/200, 500/500 and 1,000/1,000 ng/mL for MET/PIO. Calibration curves containing 10 points and a blank sample were constructed by plotting the peak area ratio of the analyte to the corresponding IS versus the concentrations. Linear regression was weighted by 1/x.

Three levels of working QC solutions were diluted from MET#2 and PIO#2 with water and acetonitrile (1:1, v/v). Appropriate amounts of working solutions of MET/PIO were spiked into blank plasma to obtain final concentrations of 2/2 ng/mL (low QC), 50/50 ng/mL (medium QC) and 800/800 ng/mL (high QC).

**Sample preparation**

The frozen (−80°C) plasma samples were thawed at 37°C, then 150 µL of acetonitrile containing IS-1 and IS-2 (50/50 ng/mL) were added to 50 µL plasma in a 1.5 mL polypropylene microcentrifuge tube. The mixture was vortex-mixed thoroughly for 2 min and then centrifuged at 20,879 x g for 10 min. The supernatant was transferred to another clean tube and centrifuged again at 30,065 x g for 5 min. An aliquot of 10 µL of the supernatant was directly injected into the HPLC–ESI-MS-MS system.

**Dilution test samples**

Dilution test samples were prepared by spiking respective working solutions into blank dog plasma. The following concentrations were used for dilution tests: 50/50, 1,000/1,000 and 16,000/16,000 ng/mL for MET/PIO.

**Data analysis and method validation**

All data were processed using Xcalibur 2.0 SUR1 software. The method was validated for specificity, linearity, sensitivity, accuracy, precision, stability, recovery and matrix effect, according to the Food and Drug Administration (FDA) guidelines for bioanalytical method validation over a concentration range of 1–1,000 ng/mL.

The stability studies of MET/PIO in dog plasma were conducted over a period of 24 h at 25°C (room temperature under laboratory light), after storage at −80°C for two months, after three freeze-thaw cycles (frozen at −80°C and thawed at 37°C) and in processed samples in the autosampler at 4°C for three days at low, medium and high concentrations (2/2, 50/50 and 1,000/1,000 ng/mL) for five replicates.

To determine recovery and matrix effects, samples at four levels (2/2, 10/10, 100/100 and 1,000/1,000 ng/mL, n = 5) were analyzed using the same assay. The relative recovery from
beagle dog plasma was determined by comparing peak area ratios (analyte/IS) of extracted analytes with those of post-extracted blank plasma spiked with MET/PIO and IS-1/2. A matrix effect may exist because of ionization competition between the analytes and co-eluent when a mass spectrometer is used as detector. The matrix effect was evaluated by comparing the peak area ratios (analyte/IS) of post-extraction blank plasma samples to those prepared in deionized water at corresponding concentrations. The matrix effect and recovery for IS-1/2 (50/50 ng/mL) were evaluated in the same way.

Pharmacokinetic study
Beagle dogs (three females, three males) were purchased from China Pharmaceutical University Laboratory Animal Center (Nanjing, China). All animal studies were approved by the Animal Ethics Committee of China Pharmaceutical University. After fasting overnight with free access to water for at least 12 h, dogs were administered a single oral dose of 1,000 mg MET/30 mg PIO in the form of ACTOplus MET XR. Blood samples were collected in 1.5 mL heparinized polythene tubes at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24 and 36 h. Plasma was centrifuged at 5,939 × g for 10 min and then transferred to clean polypropylene tubes and stored at −80°C until LC–MS–MS analysis.

Results

IS selection
Moroxydine (IS-1) and rosiglitazone (IS-2) were chosen as the ISs for MET and PIO, respectively, because they showed similar structures and retention times to the corresponding analyte. Table I compares the accuracy and precision data for the determination of MET and PIO by using their corresponding IS with those data when exchanging their IS. The results indicate that better accuracy and precision can be achieved when using two ISs than with using only one IS.

Method development
The optimum mobile phase was found to be acetonitrile–water with 6 mM ammonium acetate and 0.1% formic acid (50:50, v/v) at an isocratic flow rate of 1.0 mL/min. The LC flow was split so that approximately 0.2 mL/min entered the mass spectrometer. The retention times of MET, IS-1, PIO and IS-2 were 3.0, 2.9, 6.1 and 4.9 min, respectively, with a total run time of 8 min.

Method validation
Specificity
The representative chromatograms of blank plasma and plasma sample from a beagle dog at 0.5 h after oral dosing with test

<table>
<thead>
<tr>
<th>Concentration added (ng/mL)</th>
<th>IS</th>
<th>Concentration found (mean ± SD) (ng/mL)</th>
<th>Precision (%)</th>
<th>Accuracy (%)</th>
<th>Concentration found (mean ± SD) (ng/mL)</th>
<th>Precision (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Moroxydine (IS-1)</td>
<td>Rosiglitazone (IS-2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MET</td>
<td>2</td>
<td>2.1 ± 0.2</td>
<td>7.2</td>
<td>107.4</td>
<td>2.4 ± 0.2</td>
<td>9.3</td>
<td>121.9</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>47.0 ± 1.8</td>
<td>3.9</td>
<td>94.0</td>
<td>47.5 ± 1.4</td>
<td>2.9</td>
<td>95.0</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>1,000.1 ± 64.7</td>
<td>6.5</td>
<td>100.0</td>
<td>992.9 ± 59.6</td>
<td>6.0</td>
<td>99.3</td>
</tr>
<tr>
<td>PIO</td>
<td>2</td>
<td>2.2 ± 0.2</td>
<td>9.6</td>
<td>108.5</td>
<td>1.8 ± 0.1</td>
<td>6.5</td>
<td>90.7</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>40.1 ± 3.4</td>
<td>8.5</td>
<td>80.2</td>
<td>45.8 ± 1.3</td>
<td>2.9</td>
<td>91.6</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>951.6 ± 48.8</td>
<td>5.1</td>
<td>95.2</td>
<td>1,002.4 ± 96.3</td>
<td>9.8</td>
<td>100.2</td>
</tr>
</tbody>
</table>

Figure 1. Chemical structures: MET (A); moroxydine (B); PIO (C); rosiglitazone (D).
Tablets are shown in Figures 2A and B. The peaks of MET, IS-1, PIO and IS-2 were well separated and no significant interferences from the endogenous plasma components were observed at the retention times of the analytes and IS-1/2.

Linearity and LLOQ
The calibration curves of the two analytes were linear over the concentration range of 2–1,000 ng/mL for MET and 1–1,000 ng/mL for PIO. The typical equations were $y = 0.0164276x - 0.00586824$ ($r = 0.9995$) for MET and $y = 0.00354938x - 0.00219557$ ($r = 0.9991$) for PIO. The LLOQ values for MET and PIO were 2 and 1 ng/mL, with precision and accuracy of 10.2 and 114.2% for MET and 7.7 and 111.7% for PIO. The lowest limit of detection (LLOD) values for MET and PIO were 1 and 0.5 ng/mL. Figure 2C depicts representative chromatograms of 1 ng/mL for both MET and PIO.

Precision and accuracy
The within-batch accuracy was between 94.0 and 107.4% for MET and between 90.7 and 100.2% for PIO, whereas the

Figure 2. Representative SRM chromatograms of MET, IS-1, PIO and IS-2: a blank plasma sample (A); plasma sample with 20-fold dilution of true concentrations of MET/PIO at 77.7 and 3,525.9 ng/mL from a beagle dog at 0.5 h after oral dosing with test tablets (B); plasma sample at 1 ng/mL for both MET and PIO (C).
between-batch accuracy was between 92.4 and 99.8% for MET and between 87.3 and 99.3% for PIO.

**Dilution**

Up to 20-fold dilution of MET and PIO by blank dog plasma were tested with spiked samples at three levels (Table II). The mean accuracy for diluted samples was 105.4–110.2% for MET and 101.6–108.9% for PIO.

**Stability**

Table III summarizes the results of the short-term stability, long-term stability, freeze and thaw stability and post-preparative stability of MET and PIO. The data showed the reliable stability of MET and PIO under the investigated conditions.

**Recovery and matrix effect**

The mean extraction recoveries at four concentration levels ranged from 96.4 to 112.8% for MET and 102.1 to 104.5% for PIO, respectively (Table IV). The results indicated that the method showed high recovery. The matrix values were between 82.3 and 102.3% for MET/PIO and IS-1/2 (Table IV). No significant matrix effect was observed for the analytes.

**Application to pharmacokinetic study**

The method was applied to a pharmacokinetic study of ACTOplus MET XR tablets. The mean plasma concentration-time curves of MET and PIO are shown in Figure 3. The pharmacokinetic parameters are presented in Table V. The method was clearly adequate for determining the plasma concentration profiles of MET and PIO during the 36 h sampling period.

**Discussion**

Choosing a suitable internal standard is one of the important aspects in bioanalytical methods for obtaining accuracy. To compensate for any potentially inconsistent response due to matrix effects, the IS should have an analogous structure to the analyte and should elute close to the analyte. In this method, moroxydine and rosiglitazone were chosen as the ISs of MET and PIO, respectively, to gain accuracy and to avoid matrix effects, the results of which are shown in Table I.

### Table II

<table>
<thead>
<tr>
<th>Concentration added (ng/mL)</th>
<th>MET</th>
<th>PIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution factor (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precision (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>20</td>
<td>105.4</td>
</tr>
<tr>
<td>1,000</td>
<td>20</td>
<td>106.0</td>
</tr>
<tr>
<td>16,000</td>
<td>20</td>
<td>110.2</td>
</tr>
</tbody>
</table>

### Table III

<table>
<thead>
<tr>
<th>Concentration added (ng/mL)</th>
<th>MET</th>
<th>PIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration found (mean ± SD) (ng/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Short-term stability</td>
<td></td>
<td></td>
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<tr>
<td>Long-term stability</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freeze-thaw stability</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freeze-thaw stability</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.1 ± 0.4</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>50</td>
<td>50.0 ± 3.9</td>
<td>52.2 ± 7.0</td>
</tr>
<tr>
<td>1,000</td>
<td>973.1 ± 41.6</td>
<td>1,002.3 ± 119.9</td>
</tr>
<tr>
<td>PIO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.6 ± 0.2</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>50</td>
<td>49.7 ± 3.7</td>
<td>48.4 ± 6.3</td>
</tr>
<tr>
<td>1,000</td>
<td>1,033.4 ± 67.5</td>
<td>997.8 ± 22.5</td>
</tr>
</tbody>
</table>
Due to their reversed polarity, MET and PIO showed different chromatographic behavior on reversed-phase silica columns. For instance, the retention of MET was very poor on Agilent SB-AqC18 (150 x 2.1 mm, 3.5 μm) and Kromasil 100-5C8 (150 x 2.1 mm, 5 μm) columns. Finally, the Phenomenex Synergi POLAR-RP column was selected, on which all the four compounds performed excellently. The Synergi polar-RP is an ether-linked phenyl phase with proprietary hydrophilic end-capping that is specifically designed to maximize retention and selectivity for polar and aromatic analytes.

To achieve acceptable chromatographic peak shapes and enhance MS responses, different compositions of the mobile phase and ratios of organic phase were tested. An appropriate acidity could intensify the responses and the ratio of the organic phase and ammonium acetate could significantly affect the shape and retention of the analytes. Finally, a mobile phase of acetonitrile-water with 6 mM ammonium acetate and 0.1% formic acid (50:50, v/v) was adopted at an isocratic flow rate of 1.0 mL/min.

**Conclusion**

An improved simultaneous determination method was developed and validated for MET and PIO by LC–MS-MS in dog plasma. This optimized method utilizes only a 50 μL plasma sample and provides LLOD values of MET and PIO as low as 0.15 ng/mL. The sample pretreatment is a single-step acetonitrile protein precipitation without drying or reconstitution. The recoveries ranged from 96.4 to 112.8% for MET and 102.1 to 104.5% for PIO. The method was successfully applied to the study of the pharmacokinetics of compound MET and PIO tablets in dogs. It can be concluded that the proposed method will also be suitable for reliable therapeutic drug monitoring in clinical applications.

**Acknowledgments**

The kind help of Professor Fenzhi Sun with the revision of the paper is greatly appreciated. The study was supported by the Jiangsu Province Social Development Foundation (BE2010724), and Jiangsu Province Key Lab of Drug Metabolism and Pharmacokinetics Projects (BM2012012).

**References**


