Short Communication

An UPLC–MS/MS method for the analysis of glimepiride and fluoxetine in human plasma

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\textbf{A B S T R A C T}

A sensitive and rapid ultra performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) method was developed to determine glimepiride (GPD) and fluoxetine (FLU) in human plasma using diazepam as the internal standard (IS) simultaneously. The presented method used an Acquity UPLC BEH C18 column for chromatographic separation with tandem mass spectrometric detection on a QTrap5500 mass spectrometer operated in positive ESI mode. The mobile phase is a mixture of acetonitrile and 1% formic acid in water with gradient elution at a flow rate of 0.40 mL/min. The GPD, FLU and IS were eluted at 1.46, 1.27 and 1.39 min, respectively. The MRM transitions of m/z 491.3 → 126.3 and m/z 310.5 → 148.1 were used to quantify for GPD and FLU, respectively. The linearity of this method was found to be within the concentration range of 2.5–300 ng/mL and 0.1–20 ng/mL for GPD and FLU in human plasma, respectively. The intra- and inter-day precision (RSD\%) were less than 10.3% and accuracy (RE\%) was within ±7.3%. The matrix effect were 95.3–100.7% for GPD and FLU. GPD and FLU were sufficiently stable under all relevant analytical conditions. The method was also successfully applied to the clinical samples after a single oral dose of 2 mg GLP and 40 mg FLU in patients.

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\section{Introduction}

Glimepiride (GPD, Fig. 1), 1-[p-[2-(3-ethyl-4-methyl-2-oxo-3-pyrrylone-1-carboxamido)ethyl]phenylsulfonyl]-3-(trans-4-methylcyclohexyl)urea, is an oral sulfonylurea antihyperglycemic agent. The primary mechanism of action of GPD for lowering blood glucose appears to be dependent on stimulating the release of insulin from functioning pancreatic \( \beta \) cells. In addition, extra pancreatic effects may also play a role [1,2]. GPD is indicated for the treatment of type 2 diabetes mellitus at the recommended dosage of 1–4 mg/d for adults and adolescents in China. In vivo, GPD is metabolized by cytochrome CYP2C9 isozyme-mediated hydroxylation [3,4]. The major metabolites are the cyclohexylhydroxymethyl derivative and the carboxyl derivative. Several liquid chromatography (LC) methods have been developed for the determination of GPD in biological fluids [5–10].

Fluoxetine (FLU, Fig. 1) is currently one of the widely prescribed selective serotonin reuptake inhibitors (SSRIs) for the treatment of depression, bulimia nervosa and obsessive–compulsive disorder [11]. It is extensively metabolized in the liver, via the CYP2D6 cytochrome P450 system, to its primary active metabolite, norfluoxetine [12]. In addition, CYP2C9 also plays a vital role in the metabolism of FLU. A large interindividual variability exists in the plasma concentrations of FLU after administration of the same dose of the drug, which may be partly related to the activity of CYP2C9 [13]. Most of the methods described for analysis of FLU are based on LC, especially HPLC [12], HPLC–MS/MS [14,15] and less often GC [16,17].

When GPD and FLU are used in combination in clinic, they may have many potential drug interactions as FLU is a CYP2C9 inhibitor. Even though various methods were reported in the literature for estimation of GPD and FLU individually or in combination with other drugs, no method had been reported for simultaneous estimation of these two drugs. Ultra performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) has been evaluated as a faster and more efficient analytical tool compared with current chromatography. In the present study, we developed
a UPLC–MS/MS method for the simultaneous determination of GPD and FLU using diazepam as an internal standard.

2. Materials and methods

2.1. Chemicals and reagents

GPD (purity 98.0%), FLU (purity 98.0%), and diazepam (internal standard, IS, purity 98.0%) were obtained from Sigma (St. Louis, MO, USA). Formic acid was analytical grade and purchased from the Beijing Chemical Reagents Company (Beijing, China). Acetonitrile was of HPLC grade and were purchased from Merck Company (Darmstadt, Germany). HPLC grade water was obtained using a Milli Q system (Millipore, Bedford, USA).

2.2. UPLC–MS/MS conditions

Liquid chromatography was performed on an Acquity ultra performance liquid chromatography (UPLC) unit (Waters Corp., Milford, MA) with an Acquity BEH C18 column (50 mm × 2.1 mm, 1.7 µm particle size) and inline 0.2 µm stainless steel frit filter (Waters Corp., Milford, USA). A gradient elution was employed with the mobile phase consisted of acetonitrile (A) and water containing 1% formic acid (B) as follows: 35% A (0–0.5 min), 35–80% A (0.5–1.0 min), 80% A (1.0–2.0 min), 80–35% A (2.0–2.1 min), 35% A (2.1–4.0 min). The flow rate was 0.40 mL/min. An AB Sciex QTRAP 5500 triple quadruple mass spectrometer equipped with an electro-spray ionization (ESI) source (AB Sciex Instruments, Foster City, CA, USA) was used for mass spectrometric detection. The detection was operated in the multiple reaction monitoring (MRM) mode under unit mass resolution in mass analyzers. The dwell time was set to 18 ms for each MRM transition. The MRM transitions were m/z 491.3 → 126.3, m/z 310.5 → 148.1 and m/z 285.1 → 193.1 for GPD, FLU and IS, respectively. After optimization, the source parameters were set as follows: curtain gas, 200 kPa; nebulizer gas, 350 kPa; turbo gas, 400 kPa; ion spray voltage, 3.0 kV; and temperature, 500 °C. Data acquiring and processing were performed using analyst software (version 1.5, AB Sciex).

2.3. Standard solutions, calibration standards and quality control (QC) sample

The stock solutions of GPD (1.00 mg/mL) and FLU (1.00 mg/mL) were prepared in methanol. The stock solutions were further diluted with methanol to obtain working solutions at several different concentration levels. Calibration standards and QC samples in plasma were prepared by diluting the corresponding working solutions with blank human plasma. Final concentrations of the calibration standards were 2.5, 5, 10, 25, 50, 100, 200 and 300 ng/mL and 0.1, 0.2, 0.5, 1, 2.5, 5, 10 and 20 ng/mL for GPD and FLU in human plasma, respectively. Similarly, the concentrations of QC samples in plasma were 5, 40, and 240 ng/mL and 0.2, 2, and 16 ng/mL, respectively, for GPD and FLU. IS stock solution was made at an initial concentration of 1 mg/mL. The IS working solution (50 ng/mL) was made from the stock solution using acetonitrile for dilution. All stock solutions, working solutions, calibration standards and QCs were immediately stored at −80 °C.

2.4. Sample preparation

Before analysis, the plasma sample was thawed to room temperature. In a 1.5 mL centrifuge tube, an aliquot of 200 µL of the internal standard working solution (50 ng/mL) was added to 0.1 mL of collected plasma sample. The tubes were vortex mixed for 1.0 min. After centrifugation at 13,000 × g for 10 min, the supernatant (6 µL) was injected into the UPLC–MS/MS system for analysis.

2.5. Method validation

Before using this method to determine the clinical levels, the method was fully validated for specificity, linearity, sensitivity precision, accuracy, recovery, matrix effect and stability according the United States Food and Drug Administration (US FDA) guidelines for the validation of a bioanalytical method [18].

A specificity study is designed to investigate whether endogenous substances were observed at the retention time of analytes and IS. Specificity was evaluated by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma.

To evaluate the linearity, calibration standards of eight concentrations of GPD (2.5–300 ng/mL) and FLU (0.1–20 ng/mL) were separately extracted and assayed on three separate days. The linearity for GPD and FLU were investigated by weighted (1/x²) least-squares linear regression of peak area ratios against concentrations. The LLOQ was defined as the lowest concentration on the calibration curves.

The precision and accuracy of the method were assessed by determination of QC samples in plasma at different concentrations (5, 40, and 240 ng/mL and 0.2, 2, and 16 ng/mL for GPD and FLU in human plasma, respectively) on three separate days.

Extraction recovery experiments which showed an ability to extract the analyte from the test biological samples, were evaluated by comparing the peak area ratios of extracted QC samples with those of reference QC solutions reconstituted in blank plasma extracts. Recovery of IS (50 ng/mL) was determined similarly. Matrix effect (ME) was evaluated by comparing the peak areas of analytes (Ai) obtained from plasma samples spiked with analytes after extraction to those of the pure standard solutions (Ar) at the same concentrations. The matrix effect was calculated as ME (%) = Ai/Ar × 100. The matrix effect of IS (50 ng/mL) was evaluated in the same manner.

To ensure the reliability of the results, stability assay comprising freeze-thaw stability, short-term and long-term stability were carried out. In the protocol, sample stability was tested by analyzing QC samples after short-term (2 h and 24 h) storage at room temperature and on storage at −80 °C for 60 days. At the same time, the effect of three freeze (−80 °C)–thaw (room temperature) cycles was also investigated.

Fig. 1. The chemical structures of GPD, FLU and IS in the present study: (A) GPD, (B) FLU and (C) diazepam (IS).
3. Results and discussion

3.1. Method development

Chromatographic conditions were optimized to achieve good sensitivity and peak shape for GPD, FLU and IS, as well as a short run time. We found a sharp peak for GPD, FLU and IS with good sensitivity could be achieved using a mobile phase consisting of 35% acetonitrile and 65% water containing 1% formic acid on an Acquity UPLC BEH C18 column. The retention time of GPD was too long, which made the analytical run time more than 3 min. To shorten the analytical time, gradient elution with acetonitrile and water containing 1% formic acid was performed. After optimization, the retention times for GPD, FLU and IS were 1.46, 1.27 and 1.39 min, respectively. The advantage of this method is that a relatively larger number of samples can be analyzed in a short time, thus increasing output.

3.2. Specificity

UPLC–MS/MS chromatogram of the analytes in human plasma samples were shown in Fig. 2. The retention times of GPD, FLU and IS are 1.46, 1.27 and 1.39 min, respectively. Compared with chromatogram of blank blood sample, no interference of endogenous peaks was observed.

3.3. Linearity and sensitivity

The calibration curves were created by plotting the peak area ratios of the various analytes to internal standard versus nominal concentration of the analytes standards. The typical regression equation of these curves was calculated as follows: GPD, $y = 0.0047x + 0.0014$, $r = 0.9997$; FLU, $y = 0.0015x + 0.0135$, $r = 0.9989$. The LLOQ values, which were accepted as the lowest concentration on the calibration curves, were 2.5 ng/mL for GPD and 0.1 ng/mL for FLU, respectively.

3.4. Precision and accuracy

The intra- and inter-day precision was 11.0% or less at each QC level. The accuracy of the method was between −6.1% and 7.3% at each QC level. The results are summarized in Table 1.

3.5. Recovery and matrix effect

The recovery in plasma ranged from 90.7% to 97.5%, and 92.2% to 93.8%, respectively, for GPD and FLU. The recovery of IS (50 ng/mL) in plasma was 91.2 ± 4.1%. The matrix effect in human plasma was all between 95.3% and 100.7% for GPD and FLU at different QC levels. The matrix effect for IS (50 ng/mL) was 98.5 ± 4.3%. Results are shown in Table 2.

Table 1

<table>
<thead>
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<th>Analyses</th>
<th>Concentration added (ng/mL)</th>
<th>Intra-day precision (n=6)</th>
<th>Inter-day precision (n=6)</th>
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<tr>
<td></td>
<td></td>
<td>Mean ± SD</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>GPD</td>
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<tr>
<td></td>
<td>2.5</td>
<td>2.7 ± 0.2</td>
<td>8.3</td>
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<tr>
<td></td>
<td>5</td>
<td>4.8 ± 0.4</td>
<td>7.7</td>
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<td></td>
<td>40</td>
<td>41.5 ± 2.7</td>
<td>6.6</td>
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<tr>
<td></td>
<td>240</td>
<td>241.8 ± 8.4</td>
<td>3.5</td>
</tr>
<tr>
<td>FLU</td>
<td>0.1</td>
<td>0.09 ± 0.01</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
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<td>0.20 ± 0.02</td>
<td>8.8</td>
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<tr>
<td></td>
<td>2</td>
<td>2.1 ± 0.1</td>
<td>7.0</td>
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<tr>
<td></td>
<td>16</td>
<td>16.2 ± 0.5</td>
<td>3.2</td>
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</table>
3.6. Stability

Stability tests were performed at the low, medium and high QC samples with five determinations for each under different storage conditions. The RSDs of the mean test responses were within 15% in all stability tests.

Table 3 shows the stability data for GPD and FLU in plasma under different storage and temperature conditions. There was no effect on the quantitation for plasma samples kept at room temperature for 2 h and 24 h. No significant degradation was observed when samples of GPD and FLU were taken through three freeze (−80 °C)–thaw (room temperature) cycles. As a result, GPD and FLU in samples were stable at −80 °C for 60 days.

3.7. Patients concentrations

This validated UPLC–MS/MS method was applied successfully to 40 samples after a single oral dose of 2 mg GLP and 40 mg FLU in patients. This analytical method is suited for rapid, selective and sensitive analysis for determinations of GPD and FLU with good repeatability and accuracy in the results.

4. Conclusions

A UPLC–MS/MS method for the simultaneous determination of GPD and FLU in human plasma was developed and validated. The method affords the sensitivity, accuracy and precision needed for quantitative measurements of GPD and FLU in human plasma.

References