Simultaneous Analysis of Oral Antidiabetic Drug by LC-MS/MS

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Abstract
Diabetes is one of the most common chronic diseases, and several new oral agents have been approved for type 2 diabetes management in recent years. A combination of different classes of antidiabetic agents is often required to avoid adverse effects such as severe hypoglycemia. A potential novel combination in development involves a fixed dose single-tablet combination of pioglitazone and a dipeptidyl peptidase 4 (DPP-4) inhibitor. DPP-4 inhibitors are a relatively new class of oral antidiabetic drugs. In this study, we simultaneously analyzed eight antidiabetic drugs, including newly developed DPP-4 inhibitors, by high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (HPLC–ESI–MS/MS). The developed method was applied to analyze the active ingredients in combination drugs and to determine the concentration of antidiabetic drugs in river water. Our results indicate that three recently approved antidiabetic drugs, sitagliptin, alogliptin, and vildagliptin, are present in river water.

Keywords: Oral antidiabetic drugs; LC-MS/MS; Combination drugs; River water

1. Introduction
According to the results of the National Health and Nutrition Survey Japan 2013, 16.2% of males and 9.2% of females were diagnosed as having diabetes (hemoglobin A1c of 6.5% or more) among subjects aged 20 years or older [1]. Compared to results reported in 2006, the percentage of population receiving treatment increased. Diabetes is a major cause of chronic kidney disease, and oral antidiabetic drugs are the mainstay of therapy for most patients with type 2 diabetes. Several strategies are often required to control blood glucose levels in people with type 2 diabetes. The clinical approach begins with lifestyle modifications, including increased physical activity and diet control. Standard oral hyperglycemic regimens include single drug treatments (monotherapy) and combinations of two or three drugs from different classes. Choosing between available oral antidiabetic drugs requires consideration of their benefits as well as their adverse effects. People taking a combination of oral antidiabetic drugs have about 11% higher risk of developing hypoglycemia than people on monotherapy. Dipeptidyl peptidase-4 (DPP-4) inhibitors improve pancreatic islet function by augmenting glucose-dependent insulin secretion and decreasing elevated plasma glucagon levels [2-6]. Not only are they efficacious, they are also safe (weight neutral) and do not cause significant hypoglycemia, making it a unique class of drugs. Alogliptin is a new DPP-4 inhibitor that reduces glycosylated hemoglobin A1c (HbA1c), is weight neutral, has an excellent safety profile, and can be used in combination with oral agents and insulin. An alogliptin-pioglitazone combination is advantageous because it addresses both insulin resistance and islet dysfunction in type 2 diabetes. HbA1c reductions are significantly greater with combination treatment than with either monotherapy [7]. Various types of combination drugs, which blend two different medications in to a single pill or capsule, are now available, and more will be developed in the future. Combination drugs have many benefits, including improved compliance and reduced healthcare costs. However, if an adverse drug reaction occurs following combination drug therapy, it may be difficult to identify the active ingredient responsible. Thus, the clinical field requires a precise and sensitive method to analyze active ingredients included in combination drugs.
Recently, the excretion and accumulation of pharmaceuticals in aqueous environments has become a major concern in environment pollution [8-11]. Pharmaceuticals used for humans and animals have been discharged into groundwater, river water, and wastewater as active pharmaceuticals and metabolites. Multiple classes of pharmaceuticals, including non-steroidal anti-inflammatory drugs (NSAIDs), antivirals, antipsychotics, and antiepileptics, are detected in waste, surface, and ground water. Chronic exposure to these compounds might affect humans and other life forms. Therefore, there is a need to analyze pharmaceutical residues in aqueous environments and to evaluate their adverse effects. Martín et al. reported that the concentrations of novel antidiabetic drugs were lower than those of classic antidiabetic drugs in aqueous environmental samples, such as waste water and river water [12]. These results indicate that analytical methods are necessary for evaluating the presence of novel drugs in environmental samples.

Several methods have been published for simultaneous analysis of oral antidiabetic drugs [12-15], but none have reported a fixed dose combination of alogliptin and pioglitazone. This paper describes simultaneous analysis of eight antidiabetic drugs using HPLC-tandem mass spectrometry (LC–MS/MS). This developed method was applied to determine the concentrations of the antidiabetic drugs in river water.

2. Experimental

2.1. Chemicals and reagents

Vildagliptin was purchased from LKT Laboratories (Saint Paul, MN, USA). Alogliptin benzoate was purchased from ChemScene (South Brunswick, NJ, USA). Sitagliptin phosphate was purchased from BPS Bioscience (San Diego, CA, USA). Linagliptin was purchased from BioVision (Milpitas, CA, USA). Pioglitazone hydrochloride, mitiglinide calcium hydrate, glibenclamide, and glimepiride were purchased from Wako Pure Chemical (Osaka, Japan). LIOVEL® Combination Tablets LD and SONIAS® Combination Tablets LD were purchased from Takeda Pharmaceutical (Osaka Japan). The chemical structures of each drug are shown in Fig. 1.

Ammonium formate and formic acid were purchased from Wako Pure Chemical (Osaka, Japan). Acetonitrile, methanol, and N,N-dimethylformamide (all of chromatographic analysis grade) were purchased from Kanto Chemical (Tokyo, Japan). Oasis® HLB cartridges were purchased from Waters (Milford, MA, USA). Whatman glass microfiber filters GF/F was purchased from GE Healthcare (Buckinghamshire, UK).
2.2. Standard solution and sample preparation

The stock solutions of standards were prepared in methanol and were filtered through a 0.2-µm pore-size syringe filter. The final concentrations of stock solutions were 100 µg mL⁻¹ (pioglitazone hydrochloride, glibenclamide, and glimepiride) and 1 mg mL⁻¹ (alogliptin benzoate, linagliptin, sitagliptin phosphate hydrate, vildagliptin, and mitiglinide calcium hydrate). The standard solution, containing a 1 µg mL⁻¹ mixture of each antidiabetic drug, was prepared in methanol. This solution was diluted again with methanol to obtain the final working solutions. The antidiabetic extracting solutions of combination tablets were extracted from LIOVEL® Combination Tablets LD and SONIAS® Combination Tablets LD (Takeda Pharmaceutical, Osaka, Japan). An appropriate amount of the ground tablets was weighed and mixed with methanol (glimepiride and alogliptin) or a 9:1 mixture of methanol and 0.1 M hydrochloric acid (pioglitazone) to obtain a stock solution of 100 µg mL⁻¹ alogliptin and 60 µg mL⁻¹ pioglitazone (LIOVEL® Combination Tablets LD), 750 µg mL⁻¹ pioglitazone, and 50 µg mL⁻¹ glimepiride (SONIAS® Combination Tablets LD). The suspension was treated in an ultrasonic bath for 30 min and centrifuged at 3000 rpm for 10 min. Then, the supernatant was filtered through a 0.2-µm pore-size syringe filter. This solution was diluted again with methanol to obtain the final working solutions.

2.3. LC-MS/MS conditions

The LC-MS/MS apparatus was an ABSciex QTrap 3200® (operated in the ESI positive mode, gas 1, nitrogen (80 psi); gas 2, nitrogen (80 psi); ion spray voltage, 4500 V; ion source temperature, 500°C; curtain gas, nitrogen (45 psi); collision gas, medium) with a Shimadzu Prominence LC-system (Shimadzu LC-20 AD pumps, including a degasser, Shimadzu SIL-20 ACHT autosampler, and Shimadzu CTO-20A column oven; Shimadzu, Kyoto, Japan). Gradient elution was performed on a separation column (HITACHI LaChromUltra C18, 50 mm × 2.0 mm I.D., 2 µm; HITACHI, Tokyo, Japan). The mobile phase consisted of a mixture of methanol and 0.1 M hydrochloric acid (pioglitazone) to obtain a stock solution of 100 µg mL⁻¹ alogliptin and 60 µg mL⁻¹ pioglitazone (LIOVEL® Combination Tablets LD), 750 µg mL⁻¹ pioglitazone, and 50 µg mL⁻¹ glimepiride (SONIAS® Combination Tablets LD). The suspension was treated in an ultrasonic bath for 30 min and centrifuged at 3000 rpm for 10 min. Then, the supernatant was filtered through a 0.2-µm pore-size syringe filter. This solution was diluted again with methanol to obtain the final working solutions.

2.4. Sample collection

River samples were collected in October 2014 from the Tama River (Tokyo, Japan) and stored in dark glass bottles. Samples were taken at point 1, a location about 60 km from the river mouth on October 11, at point 2, about 40 km from the river mouth on October 17, and at point 3, about 30 km from the river mouth on October 9, 2014. Prior to extraction, samples were filtered through a glass fiber membrane filter (Whatman GF/F).

2.5. SPE procedure

Sample (1000 mL) was passed through HLB cartridge column by a continuous flow at a rate of approximately 20.0 mL/min and then washed with 10 mL of Milli-Q water. Cartridges were previously conditioned with 5 mL of methanol followed by 5 mL of Milli-Q water. The elution was performed with 5 mL of methanol by a continuous flow at a rate of approximately 1 mL min⁻¹. An extract was evaporated and then dissolved with 1 mL of mobile phase B. Method accuracy was evaluated by recovery experiments of the target compounds in spiked, real samples. Samples were spiked with a standard solution containing 1 ng mL⁻¹ each compound and were extracted using an Oasis® HLB cartridge column. The recoveries were calculated by comparing the peak area of spiked samples before extraction and that after extraction.

3. Results and discussion

3.1. LC-MS/MS analysis

The antidiabetes used in this study are shown in Fig. 1. Total ion current chromatograms of 8 antidiabetics are shown in Fig. 2, and the total analysis time was 16 min. The concentration of each antidiabetic drug was 1 µg mL⁻¹. In the MS-MS experiments, the protonated precursor molecular ions [M + H]⁺ of each analyte, vildagliptin (m/z 304), alogliptin benzoate (m/z 340), sitagliptin (m/z 408), linagliptin (m/z 473), pioglitazone (m/z 357), mitiglinide (m/z 316), glibenclamide (m/z 494), and glimepiride (m/z 491), were selected and fragmented by nitrogen gas collision. These mass spectra resulting from fragmentation were acquired in the multiple reaction monitoring (MRM) mode at m/z 154 for vildagliptin, m/z 116 for alogliptin benzoate, m/z 174 for sitagliptin, m/z 420 for linagliptin, m/z 134 for pioglitazone, m/z 126 for mitiglinide, m/z 369 for glibenclamide, and m/z 352 for glimepiride. MS/MS spectra for each antidiabetic drug are shown in Fig. 3.

3.2. Method validation

As shown in Table 1, the calibration curve was found to be linear over the concentration range of 0.1–1000 ng mL⁻¹. The correlation coefficient of the calibration curves generated during the validation was 0.999 (Table 1).
Method precision, expressed as repeatability in terms of the coefficient of variation (CV), was evaluated. The results for precision were very good as shown in Table 1. For six independent determinations at 10–1000 ng mL⁻¹, the CV value was less than 10%. The recoveries were calculated as the percentage of the analytes recovered (Table 1).

3.3. Analysis of combination drugs
LC-MS was applied to analyze combination drugs. Fig. 4A shows LC-MS analysis of alogliptin and pioglitazone in a LIOVEL® LD combination tablet. A potential novel combination in development involves a fixed dose single-tablet combination of pioglitazone and a DPP-4 inhibitor. The DPP-4 inhibitor acts mainly to increase prandial insulin secretion, while pioglitazone improves insulin sensitivity. The retention times of alogliptin and pioglitazone were found to be 5.98 and 9.20 min, respectively. This procedure was repeated for the sample solution obtained from the formulation. The formulation assay results, expressed as a percentage of the label claim, are 104.0 and 100.3%, respectively. LC-MS analysis of pioglitazone and glimepiride in a SONIAS® HD combination tablet are shown in Fig. 4B. The retention times of pioglitazone and glimepiride were found to be 9.20 and 15.99 min, and the formulation assay results were 95.4 and 95.1%, respectively. These results indicate that the amount of each drug in the tablets corresponds to the requirement of 90–110% of the label claim. Simultaneous determination of the active ingredients in combination drugs was achieved.

3.4. Determination of the antidiabetic drugs in river water
The optimized method was used to determine the concentration of the antidiabetic drugs in river water (n = 3). Samples were collected from three regions of the Tama River. A simple solid-phase extraction (SPE) technique was employed for the sample preparation in this work. No assay results, expressed as a percentage of the label claim, are 104.0 and 100.3%, respectively. LC-MS analysis of pioglitazone and glimepiride in a SONIAS® HD combination tablet are shown in Fig. 4B. The retention times of pioglitazone and glimepiride were found to be 9.20 and 15.99 min, and the formulation assay results were 95.4 and 95.1%, respectively. These results indicate that the amount of each drug in the tablets corresponds to the requirement of 90–110% of the label claim. Simultaneous determination of the active ingredients in combination drugs was achieved.

### Table 1. Calibration curve, coefficient of variation (CV) of the peak area, recovery and limits of detection (LOD).

<table>
<thead>
<tr>
<th>Antidiabetic Drug</th>
<th>Formula</th>
<th>Calibration curve</th>
<th>Recovery (%)</th>
<th>LC/MS LOD(µg/mL)</th>
<th>UHPLC* LOD(µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vildagliptin</td>
<td>y = 4171.8 x</td>
<td>R² = 0.9973</td>
<td>2.66, 2.06, 1.78, 1.57, 1.94</td>
<td>49.10</td>
<td>0.001</td>
</tr>
<tr>
<td>Alogliptin</td>
<td>y = 2105.8 x</td>
<td>R² = 0.9999</td>
<td>7.86, 6.83, 3.22, 1.33, 2.08</td>
<td>44.12</td>
<td>0.001</td>
</tr>
<tr>
<td>Sitagliptin</td>
<td>y = 1087.7 x</td>
<td>R² = 0.9999</td>
<td>6.76, 5.76, 4.78, 4.33, 3.03</td>
<td>40.10</td>
<td>0.001</td>
</tr>
<tr>
<td>Linagliptin</td>
<td>y = 970.75 x</td>
<td>R² = 0.9955</td>
<td>5.74, 4.52, 0.77, 1.83, 1.92</td>
<td>118.80</td>
<td>0.001</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>y = 21012 x</td>
<td>R² = 0.9999</td>
<td>5.19, 4.02, 2.68, 3.09, 2.15</td>
<td>92.32</td>
<td>0.001</td>
</tr>
<tr>
<td>Mitiglinide</td>
<td>y = 1732.7 x</td>
<td>R² = 0.9998</td>
<td>9.02, 5.30, 4.02, 1.92, 3.20</td>
<td>91.48</td>
<td>0.001</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>y = 3556.6 x</td>
<td>R² = 0.9999</td>
<td>9.02, 5.30, 4.02, 1.92, 3.20</td>
<td>91.48</td>
<td>0.001</td>
</tr>
<tr>
<td>Glimepiride</td>
<td>y = 2188.2 x</td>
<td>R² = 0.9998</td>
<td>9.02, 5.30, 4.02, 1.92, 3.20</td>
<td>91.48</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*Column: SHISEIDO CAPCELL CORE C18 2.7 µm (2.1 mm i.D. × 100 mm), eluent a) 10 mM HCOONH₄–CH₃CN–HCOOH (900:100:1), b) CH₃CN–HCOOH (1000:1), gradient: a+b = 0 min; 100/0→3 min; 70/30→5.5 min; 40/60, flow rate: 0.6 mL/min, Column temp.: 30°C, detection: 220 nm (DAD 200–400 nm), flow cell length: 10 mm (standard), injection vol: 10 µL.*
significant direct interference in the blank river water was observed at the retention time of the analytes. Out of 8 drugs, four were detected and confirmed: sitagliptin, alogliptin, vildagliptin, and mitiglinide. Confirmation was achieved by comparing the analyte retention times and MRM chromatograms of river water shown in Fig. 5.

In the river water at point 2, sitagliptin, alogliptin, vildagliptin, and mitiglinide were detected at mean concentrations of 13.3, 5.1, 44.1, and 2.9 ng mL\(^{-1}\), respectively, and at point 3 at mean concentrations of 23.9, 7.4, 35.0, and 5.6 ng mL\(^{-1}\), respectively. The concentration of drugs downstream are higher than those upstream. Interestingly, sitagliptin and vildagliptin detected in river water in our study, were the same drugs as previously reported in the Danube River (Linz, Austria) [12].

4. Conclusions

In this paper, we describe simultaneous and selective analysis of four classic (pioglitazone, mitiglinide, glibenclamide, and glimepiride) and four novel (vildagliptin, sitagliptin, alogliptin benzoate, and linagliptin) antidiabetic drugs. To the best of our knowledge, this is the first report on the simultaneous analysis of active ingredients including alogliptin benzoate in combination drugs and the determination of novel DPP-4 inhibitors in river water by LC-MS/MS. These findings demonstrate the significance of the analytical method, in that it is capable of detecting new antidiabetic drugs present in river water at concentrations higher than classical antidiabetic drugs.
References


