A Simple Liquid Chromatography–Tandem Mass Spectrometry Method for Determination of Plasma Fentanyl Concentration in Rats and Patients with Cancer Pain

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Abstract

A fentanyl patch is widely used for the treatment of cancer pain. Its few adverse effects include constipation and drowsiness. The absorption volume of transdermally applied fentanyl may differ according to its site of application and variability in patch adhesion. Since fentanyl is predominantly metabolized by the drug-metabolizing enzyme cytochrome P450 (CYP) 3A4 in the liver, its concentration may vary in cases of physiologically reduced CYP3A4 activity in the liver (liver disease and aging) or on co-administration of drugs. The clinical significance of measuring plasma concentration of fentanyl is high, but conventional methods require complicated processes such as solid-phase extraction and liquid–liquid extraction before the sample is injected into an HPLC system. In this study, a simple liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed for determining plasma fentanyl concentrations by deproteination with acetonitrile. A recovery test was conducted using an absolute calibration curve to confirm the method’s linearity and intra- and inter-day reproducibility. The required plasma volume for detection was reduced from 1 mL in the conventional method to 20 µL in the present study, and a good calibration curve was obtained in the concentration range from 0.05 to 5 ng/mL. These findings suggest that the method for sample preparation and quantification developed in this study are appropriate for measuring fentanyl concentration in human plasma in clinical settings.

Key words: fentanyl; liquid chromatography–tandem mass spectrometry; plasma concentration; pharmacokinetics; rat; human

Methods

Fentanyl is widely used for the treatment of cancer pain and has adverse effects such as constipation and drowsiness, which are common to other opioids, including morphine and oxycodone.1–3 Fentanyl is the only strong opioid marketed as a transdermal patch, due to features suitable for transdermal medication: high lipophilicity and small molecular weight.4 When applied to the skin, the plasma concentration of fentanyl increases gradually and remains constant over 72 h.5,6 However, the extent of drug absorption may differ according to its site of application and the variability in patch adhesion.7,8 Fentanyl is mainly metabolized to pharmacologically inactive norfentanyl by hepatic cytochrome P450 (CYP) 3A4. Its plasma concentration may therefore vary as a result of physiologically reduced CYP3A4 activity in the liver (liver disease and aging) or co-administration of drugs either inducing or inhibiting CYP3A4.9,10

As outlined above, individual differences in the plasma concentrations of fentanyl is likely to occur when applied to the skin because of the dosage form and metabolic characteristics of the drug itself.11 It is therefore inevitable to assess the precise plasma concentration of fentanyl for its proper and safe use.

The plasma concentration of fentanyl in patients treated for pain relief is about 1–2 ng/mL. Therefore, the method used to determine the plasma concentration of fentanyl requires high sensitivity to enable safe management of chronic pain.

Materials and Methods

Materials

Fentanyl (fentanyl citrate) was purchased from Daiichi-Sankyo (Tokyo, Japan) as a commercial preparation for fentanyl injections. Acetonitrile was purchased from Wako
and was evaporated to dryness under an N₂ gas stream. The fentanyl dissolved in methanol was spiked into a glass tube prepared using the following method. The desired amount of fentanyl with a concentration range of 0.05–5 ng/mL was added to plasma or human serum albumin solution containing fentanyl for deproteination, and then mixed and centrifuged (12000 × g, 10 min, 4°C) to obtain a supernatant. Aliquots of the supernatant were injected into LC system.

**LC-MS/MS Conditions**Prominence (Shimadzu, Kyoto, Japan) was used for LC system. Chromatographic separations were performed using a mobile phase composed of acetonitrile and 0.1% formic acid (30:70) with a flow rate of 0.2 mL/min. Inertsil ODS-3 (2.1×150 mm, GL Sciences, Tokyo, Japan) column was used. The column temperature was set at 40°C. The injection volume of the sample supernatant into the column was 20 μL. API 4000 tandem mass spectrometry (MS/MS) system (AB Sciex, Framingham, MA, U.S.A.) was operated with an electro-spray ionization (ESI) interface in positive ionization mode. The quantification was performed in the multiple reaction-monitoring (MRM) mode with specific ion transitions of precursor ion to product ion at m/z 337.2→m/z 188.5. The structure and fragmentation pattern of fentanyl are shown in Fig. 1. Samples were introduced to the interface through a turbo ionspray with the temperature set at 300°C. A high positive voltage of 5.5 kV was applied to the ion spray. Collision gas, curtain gas, ion source gas 1, and ion source gas 2 were set at 4.0 psi, 30 psi, 50 psi, and 80 psi, respectively.

**Validation of the LC-MS/MS Assay Method. Linearity of the Calibration Curve** An absolute calibration curve for fentanyl with a concentration range of 0.05–5 ng/mL was prepared using the following method. The desired amount of fentanyl dissolved in methanol was spiked into a glass tube and was evaporated to dryness under an N₂ gas stream. The residue was dissolved in aliquots of rat plasma to yield suitable concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2, and 5 ng/mL. The linearity of the absolute calibration curve, which was drawn from fentanyl concentration versus its peak area, was evaluated using the decision coefficient (R²).

**Accuracy and Precision** The reproducibility of the quality control sample was verified at 3 concentrations: 0.1, 0.5, and 2 ng/mL. The accuracy and precision were determined by triplicate measurements of intra-day and inter-day variations (1 week). The coefficient of variation (CV%) was calculated as the S.D./mean×100. A recovery test was performed by comparing peak height of fentanyl spiked into either the rat plasma or methanol.

**Application for Experimental and Clinical Study** The assay developed in this study using LC-MS/MS was applied to experimental and clinical pharmacokinetic studies in rats and human.

**Experimental Animal Study** Eight- to 9-week-old male Wistar rats (SLC Japan, Hamamatsu, Japan) were used for the experiment. The rats were housed under controlled environmental conditions (temperature of 23±1°C and humidity of 55±5%) with a commercial food diet and water freely available. All animal experiments were carried out in accordance with the guidelines of Meijo University for the care and use of laboratory animals.

Rats under anesthesia by intraperitoneal injection of sodium pentobarbital (30 mg/kg of body weight) were cannulated with polyethylene tubes into the right jugular vein for drug administration and blood sampling. The injection solution of fentanyl diluted with saline to a concentration of 10 μg/mL was intravenously administered into the catheter at a dose of 25 μg/kg. The blood samples (0.25 mL) were collected at designated time intervals (10, 20, 30, 45, 60, 90, 120, 150, and 180 min after administration). The collected blood samples were centrifuged (11000 × g, 10 min, 4°C) to obtain plasma that was stored at −20°C until analysis. A calibration curve for measuring rat plasma concentration of fentanyl was prepared at concentrations of 0.2, 0.5, 1, 2, 5, 10, and 20 ng/mL using rat plasma.

Plasma concentrations of fentanyl in rats receiving an intravenous injection were determined using the LC-MS/MS assay.

**Clinical Study** In the clinical study, plasma fentanyl concentrations in patients applying a transdermal fentanyl patch were measured. Blood samples were collected from a 55-year-old female patient and a 69-year-old male patient who were continuously prescribed a transdermal fentanyl patch for the treatment of cancer pain in Nagoya Ekisaikai Hospital in December 2010. Patient 1 was medicated with Fentos™ tape 1 mg and patient 2 was medicated with Durrotep™ MT Patch 4.2 mg. The amount of fentanyl contained in Fentos™ tape 1 mg and Durrotep™ MT Patch 4.2 mg are 0.64 mg and 4.2 mg, respectively. As shown in Table 1, no significant abnormality was observed in the liver or renal function in either patient. In addition, there was no combined use of a medicine metabolized by CYP3A4 or known to induce or inhibit
CYP3A4 activity.

The clinical protocol was approved by the Ethics Committee of Nagoya Ekisaikai Hospital and written informed consent was obtained from both patients.

Five-milliliter blood samples were collected from each patient at 1 h, 24 h, and 48 h after the application of transdermal fentanyl patches, using heparin as an anticoagulant agent. The collected blood samples were centrifuged (2000×g, 10 min, 4°C) to obtain plasma and were stored at −20°C until analysis. A calibration curve for measuring human plasma concentration of fentanyl was prepared for the concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2, and 5 ng/mL using a 4% human serum albumin solution.

Pharmacokinetic Analysis Plasma concentration–time data for fentanyl in each rat after a single administration were analyzed individually using a noncompartmental model. The terminal elimination rate constant (β) was calculated by determining the slope of the least-squares regression line from the terminal portion of the log concentration–time data. The area under the curve (AUC) of plasma concentration–time and the area under the first-moment curve (AUMC) were calculated by the trapezoidal method up to the last measured concentration in plasma and were extrapolated to infinity. The mean residence time (MRT) was calculated as $\text{MRT} = \frac{\text{AUMC}}{\text{AUC}}$. The systemic clearance (CLSYS) was calculated as $\text{CL}_{\text{SYS}} = \frac{\text{dose}}{\text{AUC}}$, and the steady-state volume of distribution ($V_{\text{SS}}$) was calculated as $V_{\text{SS}} = \text{CL}_{\text{SYS}} \times \text{MRT}$.

RESULTS AND DISCUSSION

Chromatography Chromatograms of the blank rat plasma and fentanyl spiked rat plasma are shown in Fig. 2. The chromatographic retention time of fentanyl was 4.2 min. There was no significant interference from endogenous compounds in the rat plasma observed at the retention time of fentanyl.

Validation of the LC-MS/MS Assay Method The linearity of the absolute calibration curve prepared from the rat plasma was confirmed by $R^2$ of 1.000 at a fentanyl concentration of 0.05–5 ng/mL (data not shown). Triplicate measurements of intra-day and inter-day variations using the fentanyl quality control samples dissolved in rat plasma are shown in Table 2. The CV% of the intra-day variation was 2.3 to 3.1%, and the recovery rate was 93.0 to 104.4%. The CV% of the inter-day variation was 0.8 to 3.1%, and the recovery rate was 91.6% to 105.3%.

The results of the recovery test suggested that plasma fentanyl could be detected without a complicated separation process by taking advantage of the characteristics of LC-MS/MS.

Fentanyl Plasma Concentration in Rats after Intravenous Administration Plasma concentration–time profiles of fentanyl after intravenous administration of 25 µg/kg in rats are illustrated in Fig. 3. The highest and lowest plasma concentrations of fentanyl were approximately 15 ng/mL and 0.4 ng/mL at 10 min and 180 min after injection, respectively. The representative pharmacokinetic parameters are summarized in Table 3.

The pharmacokinetics of fentanyl in rats has been previously reported. Choi et al. found that the peak plasma

Table 1. Demographic and Clinical Profiles of Cancer Patients in the Present Study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patient 1</th>
<th>Patient 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Age (year)</td>
<td>55</td>
<td>69</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>46</td>
<td>37</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>13</td>
<td>61</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Concomitant medication</td>
<td>Loxoprofen</td>
<td>Loxoprofen</td>
</tr>
<tr>
<td></td>
<td>Voglibose</td>
<td>Magnesium oxide</td>
</tr>
<tr>
<td></td>
<td>Glimepiride</td>
<td>Magnesium oxide</td>
</tr>
<tr>
<td></td>
<td>Sodium valproate</td>
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</tbody>
</table>

Table 2. Intra-Day and Inter-Day Variation in Recovery Yields of Fentanyl in Rat Plasma

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Intra-day</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>S.D.</td>
</tr>
<tr>
<td>0.1</td>
<td>0.093</td>
<td>0.003</td>
</tr>
<tr>
<td>0.5</td>
<td>0.465</td>
<td>0.014</td>
</tr>
<tr>
<td>2.0</td>
<td>2.089</td>
<td>0.047</td>
</tr>
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</table>
concentration of fentanyl observed at 5.18 h after intramuscular administration of 10 µg/kg was 2.1 ng/mL. They also reported that the elimination rate constant and the elimination half-life (t1/2) analyzed by the one-compartment model were 0.186 h−1 and 3.72 h, respectively. It has been demonstrated that the maximum peak concentration, the time to achieve maximum peak concentration and the elimination half-life of fentanyl in rats receiving an intraperitoneal injection of 100 µg/kg fentanyl were 21.3 ng/mL, 28.3 min and 198.3 min, respectively. On the other hand, Ohtsuka et al. have reported that the elimination half-life, total clearance and volume of distribution of fentanyl analyzed by the one-compartment model were 0.84 h, 2.46 L/h/kg and 2.98 L/kg, respectively, in male Wistar rats receiving an intravenous injection of 30 µg/kg. In the present study, 11 ng/mL of plasma fentanyl was found in rats at 10 min after an intravenous administration with a bolus intravenous dose of 25 µg/kg of fentanyl. The plasma concentration and the corresponding pharmacokinetic parameters of fentanyl determined by our LC-MS/MS method seemed to be comparable with these reports. However, the plasma-concentration time data of fentanyl showed a biphasic elimination profile and the elimination half-life of fentanyl determined in this study was somewhat shorter than those from previous reports.

**Fentanyl Plasma Concentration in Cancer Pain Patients after Transdermal Administration** The plasma concentration–time profiles of fentanyl after transdermal application in patients with cancer pain are shown in Fig. 4. The plasma concentration of fentanyl in patient 1 ranged from 0.17–0.21 ng/mL during the 48 h transdermal fentanyl patch application. In patient 2, the plasma concentration of fentanyl increased from 0.27 ng/mL at 1 h to 0.41 ng/mL at 24 h, and then decreased to 0.33 ng/mL at 48 h after the application.

It has been reported that the human plasma concentration of fentanyl reaches 0.29±0.11 ng/mL upon application of Fentos™ Tape 1 mg. In the case of the Durotep™ MT patch 4.2 mg, Kokubun et al. reported that the plasma concentration of fentanyl reached 0.54±0.27 ng/mL. Plasma fentanyl concentrations observed in the present study were comparable with the above reports.

Rapid quantitation method is required, when measuring plasma fentanyl concentration for therapeutic drug monitoring (TDM). Therefore, effective and simple pretreatment of the plasma samples is desired. Sample preparation method established in the present study allows LC-MS/MS quantitation, only by deproteinization with acetonitrile, which does not require solid-phase nor liquid–liquid extraction procedure. In conclusion, simple and rapid sample preparation method established in the present study is expected to be clinically useful, in addition to reduction of analytical time. Moreover, plasma sample volume, which corresponds to approximately 1/50 of previous reports, might be expected to relieve burden on patients during blood sampling. A small amount of blood which was collected by puncturing the fingertip or earlobe might be available for TDM of fentanyl by improvement of this method.

For clinical application, our LC-MS/MS method allows a prompt adjustment of the dosages of a transdermal patch, particularly in patients with physiologically reduced CYP3A4 activity such as hepatic disease and in patients receiving drugs changing CYP3A4 activity. It is also practical in assessing the possibility of analgesic tolerance and a ceiling effect when the desired analgesic effect cannot be achieved by a dosage increase, and helps to determine the corresponding dosage of other opioids when carrying out an opioid rotation.

**CONCLUSION**

The present study developed an LC-MS/MS method of measuring plasma fentanyl concentration at concentrations ranging from 0.05 to 5 ng/mL with a simple sample preparation using deproteinization with acetonitrile. Simplifying the purification process can reduce the analytical time and the

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean±S.D.</th>
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<tbody>
<tr>
<td>CLSYS (L/h/kg)</td>
<td>3.03±0.72</td>
</tr>
<tr>
<td>FSO (L/kg)</td>
<td>2.17±0.69</td>
</tr>
<tr>
<td>AUC (ng h/mL)</td>
<td>8.53±1.80</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>0.71±0.07</td>
</tr>
<tr>
<td>β (h−1)</td>
<td>0.74±0.10</td>
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</table>

Fentanyl at a dose of 25 µg/kg was administered. CLSYS, systemic clearance; FSO, volume of distribution at steady state; AUC, area under the plasma concentration–time curve; MRT, mean residence time; β, elimination rate constant.
required plasma sample to 20 μL. Therefore, the rapid and simple measuring method introduced in the present study could be considered a clinically favorable and effective method for determining the plasma fentanyl concentration in patients with cancer pain.

REFERENCES


6) Van Nimmen NF, Poels KL, Menten JJ, Godderis L, Veulemans L. Therefore, the rapid and simple measuring method introduced in the present study could be considered a clinically favorable and effective method for determining the plasma fentanyl concentration in patients with cancer pain.

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