Simultaneous Determination of Pethidine and Norpethidine in Mouse Plasma by Liquid Chromatography–Electrospray Ionization Source-Mass Spectrometry

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This study aims to develop an analytical method for the simultaneous determination of pethidine and norpethidine in mouse plasma using the liquid chromatography–electrospray ionization source-tandem mass spectrometry (LC–ESI-MS-MS) method. After being alkylated, the plasma specimens were extracted by liquid–liquid extraction with ethyl acetate. Chromatographic separation was performed on a C18 column (150 × 2.1 mm internal diameter, 5 µm) using a mobile phase consisting of acetonitrile/water (5 mM ammonium acetate in water, pH 6.2, 40:60, v/v) during a total run time of 4.0 min. The mass spectrometer was operated under the positive ionization mode. The multiple reaction monitoring with the transitions of m/z 248.1 → 174.2, 234.2 → 160.0 and 389.2 → 201.1 was used to quantify pethidine, norpethidine and cetirizine (internal standard), respectively. Linear calibration curves were obtained over concentration ranges of 8.24–8,440.00 ng/mL for pethidine, and 6.15–6,300.00 ng/mL for norpethidine. The intra- and interday precisions and accuracies were <15% (relative standard deviations, RSDs), and the accuracies were within the interval of 94–103% for both. The lower limits of quantification in mouse plasma were 8.24 ng/mL for pethidine and 6.15 ng/mL for norpethidine. Subsequently, the validated method was successfully applied to a pharmacokinetics study of pethidine and its metabolite, norpethidine, in mice.

Introduction

Pethidine, a synthetic narcotic analgesic with predominant μ-receptor agonist properties, is known as meperidine in the USA and widely used to relieve moderate-to-severe pain and control postanesthetic shivering (1). Pethidine is usually extensively metabolized, and norpethidine is the major metabolite via N-demethylation by the hepatic cytochrome P-450 system. Norpethidine has half the analgesic potency of pethidine but 2- to 3-fold the potency as a central nervous system excitatory agent causing agitation, tremulousness, hallucinations and convulsions compared with pethidine (2). Multiple or high doses of pethidine give rise to the accumulation of norpethidine because of its long elimination half-life (15–30 h), compared with the parent drug (2.4–4 h) (3). Patients with hepatic and renal failure are especially predisposed to its toxic effects (4). Therefore, the quantification of pethidine and norpethidine in plasma samples provides significant insight into the connection between its plasma concentration and toxicokinetic behaviors.

Several analytical methods for pethidine have been developed using gas chromatography (GC) (5, 6), high-performance liquid chromatography (HPLC) (7), liquid chromatography–tandem mass spectrometry (LC–MS-MS) (8) and gas chromatography combined with tandem mass spectrometry (GC–MS-MS) (9, 10). Al-Tamimi et al. (11) reported an LC–MS-MS method to estimate the secretion of pethidine and norpethidine into breast milk after epidural administration of pethidine to women postcesarean delivery. However, a large volume of biological fluid (~1 mL) was required, and the gradient elution employed in their study was complicated. To our knowledge, there is no report yet for the simultaneous determination of pethidine and its metabolite norpethidine in mouse plasma by LC–ESI-MS-MS. Thus, the aim of the present study was to establish a sensitive and special LC–ESI-MS-MS method with a short overall analysis time for the simultaneous quantitation of pethidine and norpethidine in mouse plasma and to make a preliminary investigation on the pharmacokinetics of pethidine and norpethidine in mice. Our method requires only 100 µL of plasma and a running time of 4 min. This simple, rapid and specific method was successfully applied to a pharmacokinetics study of pethidine and norpethidine in mice.

Experimental

Chemicals and reagents

Pethidine (purity >99.5%) and cetirizine (purity >99.7%) were obtained from the Chinese Pharmacy Biological Products Examination Institute (Beijing, China). Norpethidine was provided by ISOTEC Inc. (Miamisburg, OH, USA). Pethidine hydrochloride injections were purchased from Qinghai Pharmaceutical factory Co., Ltd. (Xining, Qinghai, China). HPLC-grade methanol and acetonitrile were obtained from Fisher Scientific Company (Emerson, IA, USA). Ultra-pure water was prepared by a Millipore Milli-Q purification system (Bedford, MA, USA). Other chemicals and reagents were all analytical grade.

Instruments and conditions

Liquid chromatography

All analyses were conducted on a Shimadzu LC system equipped with two LC-20AD pumps, SIL-20AHT autosampler, SCL-10Avp control system, DGU-20A3 on-line degasser and CTO-20AC column oven (Chiyoda-Ku, Kyoto, Japan). Separation was performed on a Dimonsil C18 column (150 mm × 2.1 mm i.d., 5 µm, Dikma, Beijing, China) equipped with a phenomenex guard column (5.0 mm × 2.0 mm i.d., Phenomenon, Guangzhou, China). The mobile phase consisted of acetonitrile and water containing 5 mM ammonium acetate (pH 6.2, 40:60, v/v). The temperature was maintained at 30°C for the column and 15°C for the autosampler. The flow rate was 0.4 mL/min. The
chromatographic run time of each sample was 4.0 min, and the injection volume was 10 μL.

**Mass spectrometry**

Mass spectrometric analyses were conducted on an API 3,200 LC–MS-MS system (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization source (ESI) in the triple-quadrupole mode. The curtain gas and collision activated dissociation were 20 and 5 psi, respectively. The other working parameters were set as follows: spray voltage, 4,500 V; source temperature, 500°C; GAS1, 40 psi and GAS2, 20 psi. The declustering potential, entrance potential, collision energy and collision cell exit potential were optimized, respectively, as 42, 10, 25 and 3 V for pethidine; 40, 6, 20 and 2.5 V for norpethidine; 38, 5, 25 and 3.7 V for cetirizine. LC–ESI-MS-MS was performed in the positive ionization mode with multiple reaction monitoring (MRM) of the transitions m/z 248.1 → 174.2 for pethidine, m/z 234.2 → 160.0 for norpethidine, and m/z 389.2 → 201.1 for cetirizine. Data acquisition and analysis were controlled using the Analyst 1.5 software (Applied Biosystems).

**Calibration standards and quality control samples**

Primary stock solutions of pethidine (2 mg/mL), norpethidine (1 mg/mL) and cetirizine (20.72 μg/mL) were prepared in methanol. A series of working standard mixture was prepared by diluting the stock solutions to obtain the desired concentrations of 82.42, 164.84, 329.69, 659.38, 1,318.75, 2,575.00, 10,550.00, 21,100.00, 42,200.00, and 84,400.00 ng/mL for pethidine and 61.52, 123.05, 246.09, 492.19, 984.38, 3,937.50, 7,875.00, 15,750.00, 31,500.00 and 63,000.00 ng/mL for norpethidine. Internal standard (IS) working solution was prepared at 499.40 ng/mL by diluting the stock standard with methanol. All solutions were stored at −20°C when not used.

Matrix-matched calibration standard samples were prepared by adding 10 μL of working standards of pethidine and norpethidine at different concentrations into 100 μL of drug-free mouse plasma. The final concentrations were 8.24, 16.49, 32.97, 65.94, 131.88, 527.50, 1,055.00, 2,110.00, 4,220.00, 8,440.00 ng/mL for pethidine and 6.15, 12.30, 24.61, 49.21, 98.44, 393.75, 787.50, 1,575.00, 3,150.00, 6,300.00 ng/mL for norpethidine. Quality control (QC) samples of low, medium and high concentration levels of 16.49, 527.50 and 4,220.00 ng/mL for pethidine and 12.30, 394.00 and 3,150.00 ng/mL for norpethidine were prepared in a manner similar to that used for the preparation of the calibrator samples.

**Sample preparation**

A 100-μL aliquot of mouse plasma was added into 10-mL glass test tube and fortified with 20 μL of IS working solution, followed by the addition of 50 μL of 0.1 mol/L sodium carbonate solution. Then, liquid–liquid extraction (LLE) was performed by adding 1.5 mL of ethyl acetate, and the samples were vortex mixed for 4 min, followed by centrifugation at 3,000 rpm for 5 min. The supernatant was transferred into a 10-mL glass tube and dried under nitrogen stream at 40°C. The dried residue was reconstituted in 200 μL of mobile phase, and a 10-μL aliquot was injected into the LC–ESI-MS-MS system.

**Method validation**

**Linearity and limit of quantification**

A 10-point calibration curve was obtained by analyzing the matrix-matched calibration standards prepared according to the procedure described and plotting the gradient of the IS peak–area ratio versus the pethidine or norpethidine concentration of the standards. The equations of the calibration curves were fitted by least-linear regression using 1/x² weighting scheme to minimize the deviation of back-calculated values from nominal concentrations.

The sensitivity of the method was expressed as the limit of quantitation, defined as the lowest concentration on the calibration curve. Each LLOQ sample should be obtained with an acceptable accuracy (error) of 80–120% and a precision (expressed as relative standard deviation, RSD) not exceeding 20% to validate the method.

**Precision, accuracy and recovery**

The inter- and intraday accuracies and precisions of the assay were assessed by analyzing five replicates of QC samples at three concentration levels for pethidine and norpethidine within a day. The interday accuracy and precision were estimated by determining the QC samples at three concentration levels for pethidine and norpethidine on 3 consecutive days. The intra- and interday precisions were defined as the RSD. The accuracy was calculated as the percentage (measured concentration/nominal spiked concentration) × 100.

Absolute extractions were determined by comparing the peak areas of pethidine and norpethidine obtained from five replicates at three QC concentrations with those from samples prepared by extracted blank plasma samples with the same amounts of analytes.

**Matrix effect**

The degree of matrix effect was examined by comparing peak areas of pethidine- and norpethidine-fortified drug-free plasmas after solvent extraction (A) with those of analytes in the same amount of mobile phase (B). The ratio (A/B × 100%) was used to evaluate the matrix effect. The same treatment was performed for the IS.

**Stability**

Short-term, long-term and three freeze–thaw stabilities of pethidine and norpethidine were evaluated using QC samples containing known concentrations of 16.49, 527.50 and 4,220.00 ng/mL of pethidine and 12.30, 394.00 and 3,150.00 ng/mL of norpethidine. The autosampler stability was tested by analyzing processed QC samples in the autosampler at 15°C for 12 h. Short-term and long-term stabilities were assessed using untreated QC samples kept at ambient temperature for 12 h and stored at −80°C for 30 days. The freeze–thaw stabilities of the analytes were determined over three freeze–thaw cycles. In each freeze–thaw cycle, the samples were frozen and stored at −20°C for 24 h, then thawed at room temperature. Samples were considered to be stable if their assay values were within 15% error of the nominal values.
Application of the method in pharmacokinetic experiments

BABL/c mice (male, weighing 18–22 g) were obtained from the Experiment Animal Center of Tongji Medical College, Huazhong University of Science and Tongji (Wuhan, China) and kept in an environmentally controlled breeding room for at least 1 week before the experiment.

The method was successfully applied to the simultaneous determination of pethidine and norpethidine in plasma after the intraperitoneal injection of a single dose of 20 mg/kg pethidine hydrochloride. After an overnight fast, the mice (n = 84) were given a single intraperitoneal injection of 20 mg/kg pethidine hydrochloride. At 0.08, 0.17, 0.25, 0.33, 0.50, 0.75, 1, 1.5, 2, 2.5, 3 and 4 h postdosing, blood was collected into heparin-coated tubes from every seven mice. The blood samples were centrifuged at 3,000 rpm for 15 min to obtain plasma. The plasma samples were immediately frozen and stored at −80°C until analysis.

Pharmacokinetic parameters were estimated using a compartmental model via the proprietary DAS (Drug and Statistics) computer software package (Chinese Pharmacology Society). The terminal elimination rate constant (kₑ) was estimated by linear regression analysis of the terminal portion of the log-linear blood concentration–time profile. The terminal elimination half-life (t₁/₂) was calculated from the terminal elimination rate constant using the formula t₁/₂ = 0.693/kₑ. The mean peak drug concentration (Cₘₐₓ) and the time to reach Cₘₐₓ (Tₘₐₓ) were derived directly from the individual blood levels. The area under the concentration–time curve from dosing to the end of the dosing interval (AUC₀–t) was calculated by the linear trapezoidal rule. The apparent oral clearance (CL/F) was calculated from dose/AUC₀–t. The apparent volume of distribution (V/F) was calculated from dose/AUC₀–t kₑ.

Results and discussion

**Method development**

To optimize mass spectrometric conditions, the solutions containing pethidine, norpethidine and IS were individually injected into the mass spectrometer at a flow rate of 10 µL/min via a syringe pump, respectively. Mass spectrometry (MS) scans were carried out in positive and negative ionization modes, and the positive ionization mode produced higher sensitivity and fewer fragments for pethidine and norpethidine than the negative ionization mode. Similar to the previous reports (9–11), protonated molecular ions for pethidine (m/z 248.1), norpethidine (m/z 234.2) and cetirizine (m/z 389.2) were chosen as precursor ions. From the product ion scan (MS2), it could be identified that the fragment ions with m/z 174.2 for pethidine, 160.0 for norpethidine and 201.1 for cetirizine were most abundant (Figure 1). Thus, we selected the transitions m/z 248.1 → 174.2 for pethidine, 234.2 → 160.0 for norpethidine and 389.2 → 201.1 for cetirizine.

To optimize the composition of mobile phase, various combinations of methanol, acetonitrile and water were assessed to achieve the ideal peak separation. Then, to improve the peak shape, different concentrations and compositions of typical volatile buffers such as formic acid and ammonium acetate commonly used in the positive ionization mode were assessed. The results showed that the addition of ammonium acetate resulted in a better peak shape than formic acid, whereas the peaks for both pethidine and norpethidine became broad with the increase of ammonium acetate concentration. Therefore, a mixture of acetonitrile and water containing 5 mM of ammonium acetate at the ratio of 40:60 (v/v) was chosen as a mobile phase in this study, which achieved good peak shapes as well as high sensitivity for pethidine, norpethidine and IS. Meanwhile, different types and length of columns (Dimonsil C₈, Dimonsil C₁₈, Ultimate XB-CN) were evaluated, and the Dimonsil C₁₈ column provided the best chromatographic separation. The retention times of pethidine, norpethidine and IS were 1.61, 2.26 and 3.01 min, respectively, shortening the analysis time greatly compared with previous reported methods (8).
Because both pethidine and its metabolite were weak organic bases, a proper volume of 0.1 mol/L sodium carbonate was added to the samples prior to LLE to maximize the conversion of pethidine and norpethidine to the unionized form, and 50 μL of 0.1 mol/L sodium carbonate was selected. Several organic solvents were tested to maximize extraction recovery and minimize the matrix effect. High recoveries (≥81%) for both analytes were achieved when ethyl acetate (ethyl acetate versus plasma, 15:1, v/v) was used. The results showed that no interfering peaks from endogenous substances were detected at the corresponding retention times of the analyte and IS (Figure 2).

The use of ISs can minimize any variation produced in the analytical process. In this study, we tried imidapril, carbamazepine, omeprazole and cetirizine, which can be easily obtained, for IS selection. Cetirizine was selected as the IS for its high sensitivity and similar chromatographic and mass spectrometric behavior to the analytes.

Table I
Precision, accuracy of pethidine and norpethidine in mouse plasma (N = 5)

<table>
<thead>
<tr>
<th>Concentrations (ng/mL)</th>
<th>Intraday</th>
<th>Interday</th>
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<tr>
<td></td>
<td>Concentration (ng/mL)</td>
<td>RSD (%)</td>
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<tr>
<td>Pethidine</td>
<td>8.24</td>
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<tr>
<td></td>
<td>16.49</td>
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<td></td>
<td>527.50</td>
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<td></td>
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<td>Norpethidine</td>
<td>6.15</td>
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</tr>
<tr>
<td></td>
<td>12.30</td>
<td>1.05</td>
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<td></td>
<td>394.00</td>
<td>31.01</td>
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<td></td>
<td>3,150.00</td>
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Table II
Matrix effect of pethidine and norpethidine in mouse plasma (N = 5)

<table>
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<th>Concentrations (ng/mL)</th>
<th>Matrix effect</th>
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<tr>
<td></td>
<td>Matrix effects (%)</td>
</tr>
<tr>
<td>Pethidine</td>
<td>99.84 ± 3.50</td>
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<td></td>
<td>103.47 ± 2.50</td>
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<td></td>
<td>95.39 ± 4.11</td>
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<tr>
<td>Norpethidine</td>
<td>97.13 ± 6.08</td>
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<tr>
<td></td>
<td>98.73 ± 3.70</td>
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<td></td>
<td>99.04 ± 7.09</td>
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Figure 2. Representative MRM chromatograms; (A) blank mouse plasma; (B) LLOQ of pethidine (8.24 ng/mL), norpethidine (6.15 ng/mL) and IS (499.4 ng/mL) spiked in blank mouse plasma; (C) mouse plasma 1 h after intraperitoneal injection of 20 mg/kg pethidine hydrochloride. Retention times for norpethidine (1), pethidine (2) and IS (3) were 1.61, 2.26 and 3.01 min, respectively.
Method validation

Linearity and range
The calibration curves for both analytes in mouse plasma showed good linearity over the range of 8.24–8,440.00 ng/mL for pethidine or 6.15–6,300.00 ng/mL for norpethidine. Typical equations for the calibration curves were as follows: \( Y = 0.0206X + 0.008 \) for pethidine and \( Y = 0.0332X + 0.227 \) for norpethidine. The LLOQ of the analytes were 8.24 ng/mL for pethidine and 6.15 ng/mL for norpethidine in mouse plasma, with acceptable accuracy and precision (Table I). These LLOQs were sensitive enough to allow the pharmacokinetic investigation of pethidine and norpethidine following intraperitoneal injection of pethidine.

Specificity and selectivity
The specificity and selectivity of the method were verified by the lack of interfering peaks for endogenous substances in blank matrices. Representative MRM chromatograms of blank mouse plasma; blank mouse plasma fortified with an LLOQ of pethidine, norpethidine and IS; and mouse plasma are shown in Figure 2. There was no significant interference from endogenous plasma constituents at retention times of pethidine, norpethidine and IS.

Precision and accuracy
The intra- and interday RSDs and accuracies data are summarized in Table I. The intra- and interday RSDs were between 3.46 and 10.93% for pethidine and between 4.53 and 8.65% for norpethidine. The accuracy ranged from 95.85 to 102.24% for pethidine and from 94.71 to 101.91% for norpethidine. The precision did not exceed 15%, and the accuracy was within ±15% of the target concentration. All precision and accuracy results indicated that the present LC–ESI-MS-MS method was accurate and reproducible for the measurement of pethidine and norpethidine in mouse plasma.

Recovery and matrix effect
The extraction recoveries of pethidine and norpethidine in mouse plasma determined from five replicate samples at three QC levels were 90.20 ± 5.46, 88.80 ± 5.72 and 85.94 ± 2.60%, and that for norpethidine were 82.35 ± 7.42, 81.44 ± 4.07 and 83.56 ± 4.75%, with RSD% <6.44% for pethidine and <9.01% for norpethidine, respectively. The recoveries of pethidine and norpethidine were all >80% and consistent throughout the dynamic range for both analytes.

The absolute matrix effect values for pethidine and norpethidine at three QC levels were all within 85–115% (Table II).

Stability
The stabilities of pethidine and norpethidine under different storage and handling conditions were fully evaluated by analyzing QC samples. The results of stabilities are summarized in Table III. The relative errors of all samples were within 9.00%, indicating that analytes were stable under the above conditions. So, it meant a good stability for the analytes over all steps of the determination.

Application to the pharmacokinetic study
The validated method was successfully employed to determine the plasma concentration–time data. Based on the akaike information criterion value, a single compartment model was selected as the model of best fit. The mean plasma concentration–time curves were shown in Figure 3, and pharmacokinetic parameters of pethidine and norpethidine are summarized in Table IV, respectively. Plasma concentration–time profiles showed that both pethidine and norpethidine reached peak concentration at ~0.2 h after injection, indicating that pethidine was rapidly metabolized in liver by N-demethylation to norpethidine. It was also found that \( C_{\text{max}} \) of norpethidine was comparable with that of pethidine.
Conclusion

A sensitive, simple and rapid LC–ESI-MS-MS assay for the simultaneous determination of pethidine and norpethidine in mouse plasma was developed and validated with respect to sensitivity, accuracy, precision and reproducibility. Calibration curves were linear over a wide range of 8.24–84,400.00 ng/mL for pethidine and 6.15–6,300.00 ng/mL for norpethidine. The validated method was successfully applied to pharmacokinetic studies of pethidine and its metabolite norpethidine. This method may also provide an ideal reference for clinical pharmacokinetic studies of pethidine and norpethidine.

Table IV

Main pharmacokinetic parameters of pethidine and norpethidine after a single intraperitoneal administration of pethidine (20 mg/kg) in mice (mean ± SD, n = 7)

<table>
<thead>
<tr>
<th></th>
<th>T_{\text{max}} (h)</th>
<th>C_{\text{max}} (ng/mL)</th>
<th>AUC_{0–\text{t}} (ng h/mL)</th>
<th>CL/F (L/h)</th>
<th>V/F (L/kg)</th>
<th>T_{1/2z} (h)</th>
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<tbody>
<tr>
<td>Pethidine</td>
<td>0.21</td>
<td>2,512.93</td>
<td>1,295.65</td>
<td>15.92</td>
<td>59.87</td>
<td>1.10</td>
</tr>
<tr>
<td>Norpethidine</td>
<td>0.24</td>
<td>1,782.92</td>
<td>2,518.94</td>
<td>7.90</td>
<td>12.68</td>
<td>1.96</td>
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</table>

References