Pharmacokinetic Study of Nicotine and Its Metabolite Cotinine to Clarify Possible Association between Smoking and Voiding Dysfunction in Rats Using UPLC/ESI-MS

Satomi O NOUE*, Noriyuki YAMAMOTO, Yoshiki SETO and Shizuo YAMADA
Department of Pharmacokinetics and Pharmacodynamics and Global Center of Excellence (COE) Program, School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka, Japan

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Summary: The present study was undertaken to clarify the possible association between nicotine intake/cigarette smoking and detrusor instability. For pharmacokinetic characterization of nicotine and cotinine (a major and pharmacologically less active metabolite of nicotine), a rapid ultra-performance liquid chromatography/electrospray ionization-mass spectrometry (UPLC/ESI-MS) method was developed that requires only a small amount of sample and simple pretreatment. The UPLC/ESI-MS method was validated with a focus on specificity, sensitivity (limit of detection, 2.5 ng/mL; limit of quantification, 5 ng/mL), linearity ($r > 0.998$), accuracy (97.2–102.8%), precision (relative standard deviation <8%) and robustness in accordance with ICH guidelines (Q2B Validation of Analytical Procedures: Methodology). The developed method was successfully applied to determine nicotine and cotinine levels in rat biological samples such as plasma, urine and several tissues. After subcutaneous administration of nicotine ditartrate (2 mg/kg of body weight) in rats, the absorbed nicotine was rapidly and extensively metabolized into cotinine. However, nicotine was found to be predominant in cortex and bladder, where nicotinic acetylcholine receptors were expressed for neuronal control of voiding function. Repeated administration of nicotine led to a ca. 3-fold higher accumulation of nicotine than that of cotinine in rat urine. The results of the pharmacokinetic study using the UPLC/ESI-MS method further support the possible involvement of nicotine in increased risk of urinary dysfunction in smokers.

Keywords: nicotine; cotinine; UPLC; urinary excretion; cigarette smoking

Introduction

Cigarette smoke is a complex aerosol consisting of 92% gaseous components and 8% particulates, and side stream and direct stream smoke are similar in composition. The inhalation of cigarette smoke either directly by cigarette smokers or indirectly by people in the same enclosed areas represents an environmental health problem for millions of people. Cigarette smoking has long been identified as a major causative factor in the development of inflammatory lung diseases such as chronic bronchitis, pulmonary fibrosis, emphysema and chronic obstructive pulmonary disease. In addition to these respiratory diseases, cigarette smoking has also been causally linked with obesity, osteoporotic fractures, cardiovascular diseases, neurodegenerative diseases and bladder dysfunctions. In particular, recent attention has been drawn to the increased risk of urinary dysfunction in female smokers. Previous population-based studies also demonstrated cigarette smoking to be associated with both urinary incontinence and urgency in elderly people, although the evidence was conflicting. In general, several factors such as weakening of the pelvic floor muscles and collagen tissue alterations through aging and childbirth may contribute to both conditions, and these urinary dysfunctions have a substantial influence on quality of life and activities of daily living.

Although little is known about the pathogenesis of smoking-related voiding dysfunction, detrusor instability in female smokers may be related to an anti-estrogenic hormonal effect on the bladder or urethra, an adverse effect
on collagen synthesis\textsuperscript{10} and an increased turnover of vitamin C.\textsuperscript{11} It is also well established that nicotine plays an important role in the control of bladder function, mediated by nicotinic acetylcholine receptors in both central and peripheral nervous systems.\textsuperscript{12–14} In this context, nicotine in cigarette smoke might also lead to detrusor instability via neuronal control of urinary bladder responses.\textsuperscript{15} Better understanding of the pharmacokinetic behavior of nicotine may identify the involvement of nicotine in detrusor instability in smokers; however, it has never been fully elucidated.

Many analytical methods have been developed for the analysis of nicotine and its metabolites, including gas chromatography,\textsuperscript{16} high-performance liquid chromatography (HPLC),\textsuperscript{17,18} enzyme-linked immunosorbent assay (ELISA),\textsuperscript{19} and radio-immunoassay (RIA).\textsuperscript{20} The present study was undertaken to develop a more rapid analytical method for pharmacokinetic characterization of nicotine and cotinine, a major metabolite of nicotine, using ultra-performance liquid chromatography (UPLC)/electrospray ionization (ESI)-MS. The UPLC/ESI-MS method for nicotine was validated with a focus on linearity, accuracy, precision, assay recovery and robustness. Plasma and urine concentrations of nicotine and cotinine after subcutaneous injection were monitored using the developed UPLC/ESI-MS method, and the tissue distribution of these chemicals was also assessed.

**Materials and Methods**

**Chemicals:** Ammonium acetate, nicotine ditartrate and diethyl ether were purchased from Wako Pure Chemical Industries (Osaka, Japan). Cotinine, antipyrine and sodium pentobarbital were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (HPLC grade) and Millex-LG membrane filters (pore size: 0.2 µm) were bought from Kanto Chemical (Tokyo, Japan) and Millipore (Bedford, MA, USA), respectively.

**UPLC/ESI-MS analysis:** The amounts of nicotine and cotinine in biological samples from rats were determined by an internal standard method using a Waters Acquity UPLC system (Waters, Milford, MA), which included a binary solvent manager, sample manager, column compartment and SQD connected with MassLynx software. An Acquity UPLC BEH C 18 column (particle size: 1.7 µm, column size: 2.1 mm × 50 mm; Waters) was used, and the column temperature was maintained at 40°C. The standard and samples were separated using a gradient mobile phase consisting of acetonitrile (A) and 5 mM ammonium acetate (B) with a flow rate of 0.25 mL/min. The gradient condition of the mobile phase was 0–0.5 min, 5% A; 0.5–3.5 min, 5–25% A; 3.51–4.5 min, 95% A; and 4.51–5.5 min, 5% A. Analysis was carried out using selected ion recording (SIR) for \textit{m/z} 163 for nicotine [M + H]\(^+\), 177 for cotinine [M + H]\(^+\) and 189 for antipyrine [M + H]\(^+\), an internal standard. Peaks for nicotine, cotinine and antipyrine were detected at retention times of 1.90, 2.30 and 3.26 min, respectively.

**Method validation:** The newly developed UPLC/ESI-MS method was validated in terms of linearity, accuracy, precision and assay recovery according to International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines “Q2B Validation of Analytical Procedures: Methodology”. Assay method precision was investigated using six independent test solutions and a standard preparation. The intermediate precision of the assay was also evaluated using different analysts on three different days. The accuracy of the assay was evaluated in triplicate using three concentration levels: 10, 300 and 600 ng/mL. The limit of detection (LOD) and limit of quantification (LOQ) for nicotine and cotinine were estimated by injecting a series of dilute solutions with known concentration. The LOD and LOQ were estimated from standard deviation values of replicate responses of tested chemicals (signal-to-noise ratios, 3:1 for LOD and 10:1 for LOQ). To determine the robustness of the method, experimental conditions were purposely altered and the peak response for each tested chemical was examined by injecting system suitability solution. The flow rate was changed to 0.20 and 0.30 mL/min. The column temperature was varied by (±3°C), and the organic strength was varied by (±2%)%

**Pharmacokinetic study:**

**Animals**

Male Sprague–Dawley rats, weighing ca. 650 g (n = 4 for each \textit{in vivo} experiment, 25–30 weeks of age; Japan SLC, Shizuoka, Japan), were housed two per cage in the laboratory with free access to food and water, and maintained on a 12-h dark/light cycle in a room with controlled temperature (24 ± 1°C) and humidity (55 ± 5%). All procedures used in the present study were conducted in accordance with the guidelines approved by the Institutional Animal Care and Ethical Committee of the University of Shizuoka.

**Plasma concentration**

Rats were anesthetized using pentobarbital (50 mg/kg body weight) and then a guide cannula (PUC-40, EICOM, Kyoto, Japan) was inserted into the jugular vein on the day before nicotine ditartrate (2 mg/kg body weight) was administered subcutaneously. Blood samples (approximately 500 µL) were collected from the cannulated jugular vein at the indicated times (0.25, 0.5, 1, 2, 3, 4, 5, 6, 8, 10 and 12 h) after administration of nicotine ditartrate and mixed with 1,250 µL of acetonitrile containing 0.053 mM antipyrine. The mixture was centrifuged (10,000 × g, 10 min, 4°C) and the supernatant was dried with a stream of nitrogen gas. Each dried sample was redissolved in 100 µL of 50% acetonitrile solution and filtered with Millex-LG membrane filter (Millipore, Bedford, MA, USA) for UPLC/ESI-MS analysis.

**Urine concentration**

After single subcutaneous administration of nicotine ditartrate (2 mg/kg body weight), urine samples were
collected every hour. To monitor urine excretion after repeated subcutaneous administration of nicotine ditartrate [2 mg/kg body weight, b.i.d., 10 days], urine samples were collected every 12 h. Urine samples (30 µL) were mixed with 1,470 µL of acetonitrile, and the mixture was centrifuged (10,000 × g, 10 min, 4°C). Twenty microliters of 0.53 mM antipyrine was added to 180 µL of each supernatant, and the mixture was filtered with a Millex-LG membrane filter (Millipore) for UPLC/ESI-MS analysis.

**Tissue distribution**

At 0.5 and 2 h after oral subcutaneous administration of nicotine ditartrate (2 mg/kg body weight), rats were sacrificed by taking blood from the descending aorta under temporary anesthesia with diethyl ether, and the tissues were then perfused with cold saline from the aorta. The bladder, salivary gland, heart and cortex were dissected, and temporary anesthesia with diethyl ether, and the tissues were then minced with scissors and homogenized in a Physcotron (Microtech, Chiba, Japan) in 9 times its weight of ice-cold saline. A total of 400 µL of acetonitrile was added to 100 µL of homogenate, and the mixture was centrifuged (10,000 × g, 10 min, 4°C). Twenty microliters of 0.53 mM antipyrine was added to 180 µL of each supernatant, and the mixtures were filtered with a Millex-LG membrane filter (Millipore) for UPLC/ESI-MS analysis. The tissue-to-plasma concentration ratio ($K_p$ value) was calculated as the ratio of the tissue concentration of unchanged drug to the plasma concentration.

**Statistical analysis**

For statistical comparisons, one-way analysis of variance (ANOVA) with pairwise comparison by Fisher’s least significant difference procedure was used. A $P$ value of less than 0.05 was considered significant for all analyses.

**Results and Discussion**

**Method development and validation:** A UPLC/ESI-MS method that can be conveniently employed for pharmacokinetic profiling was developed for determination of nicotine and cotinine. The chromatographic conditions were adjusted to provide optimal performance of the assay, and the mobile phase for the analysis was selected on the basis of its polarity. Nicotine and cotinine were separated on a UPLC BEH C18 column using a linear gradient of acetonitrile and 5 mM ammonium acetate, which has the advantage of lower solvent consumption and increased mass sensitivity. Various sample treatment procedures were evaluated, including solid-phase extraction, protein precipitation and liquid-liquid extraction. Considering recovery for all analytes, a simple protein precipitation procedure was developed with acetonitrile, yielding over 80% extraction recovery for nicotine and cotinine. Nicotine and cotinine standard solutions were found to be stable in the mobile phase for at least 24 h at room temperature. The recovery proportions of these standard solutions stored for 24 h at room temperature were calculated to be 99.4% and 100.5% for nicotine and cotinine, respectively, when compared with freshly prepared solutions.

The specificity of the analytical method was demonstrated by comparing selected ion recording (SIR) chromatograms of nicotine, cotinine and antipyrine, an internal standard, for a blank plasma sample and a spiked plasma sample. All the analytes and the internal standard could be detected on their own selected ion chromatograms without any significant interference. Calibration graphs were constructed at five concentration levels ranging from 10 to 600 ng/mL, and both nicotine and cotinine showed good linearity with a correlation coefficient ($r$) value greater than 0.998 (data not shown). The precision of the method was determined by repeatability (intra-day), and the intermediate precision (inter-day) is expressed as relative standard deviation (RSD, %) of a series of measurements. The experimental values obtained for the determination of nicotine and cotinine in plasma are presented in Table 1. The results show an RSD of 7.2% at 10 ng/mL nicotine, and the intra-day variability was calculated from assays on 3 days and shows a mean RSD of 6.1%. The accuracy of the method was determined and the mean recovery was found to be over 98%, indicating an agreement between the true value and the value found.

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**Table 1. Accuracy and precision of nicotine and cotinine measurement in rat plasma**

<table>
<thead>
<tr>
<th>Spiked concentration (ng/mL)</th>
<th>Measured concentration (ng/mL)</th>
<th>Accuracy (%)</th>
<th>Precision (RSD, %)</th>
<th>Measured concentration (ng/mL)</th>
<th>Accuracy (%)</th>
<th>Precision (RSD, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-day</td>
<td>Inter-day</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>9.7 ± 0.7</td>
<td>97.2</td>
<td>7.2</td>
<td>9.8 ± 0.6</td>
<td>98.1</td>
<td>6.1</td>
</tr>
<tr>
<td>300</td>
<td>308.4 ± 12.7</td>
<td>102.8</td>
<td>4.1</td>
<td>295.6 ± 10.4</td>
<td>98.5</td>
<td>3.5</td>
</tr>
<tr>
<td>600</td>
<td>607.8 ± 16.4</td>
<td>101.3</td>
<td>2.7</td>
<td>609.7 ± 18.9</td>
<td>101.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Cotinine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>9.6 ± 0.5</td>
<td>96.4</td>
<td>5.6</td>
<td>10.2 ± 0.7</td>
<td>102.0</td>
<td>6.9</td>
</tr>
<tr>
<td>300</td>
<td>306.4 ± 11.2</td>
<td>102.1</td>
<td>3.7</td>
<td>303.4 ± 14.3</td>
<td>101.1</td>
<td>4.7</td>
</tr>
<tr>
<td>600</td>
<td>612.2 ± 18.1</td>
<td>102.0</td>
<td>3.0</td>
<td>592.2 ± 20.2</td>
<td>98.7</td>
<td>3.4</td>
</tr>
</tbody>
</table>

*Data represent mean ± SD (n = 6). Percentage relative standard deviation.*
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Table 2. Pharmacokinetic parameters of nicotine and cotinine following subcutaneous administration of nicotine ditartrate (2.0 mg/kg body weight of rats)

<table>
<thead>
<tr>
<th>Substance</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (ng/mL)</th>
<th>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</th>
<th>AUC&lt;sub&gt;0→∞&lt;/sub&gt; (ng h/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>122 ± 17.2</td>
<td>1.05 ± 0.190</td>
<td>103 ± 4.50</td>
</tr>
<tr>
<td>Cotinine</td>
<td>226 ± 17.6</td>
<td>7.08 ± 0.400</td>
<td>1940 ± 88.1</td>
</tr>
</tbody>
</table>

C<sub>max</sub>, maximum concentration; T<sub>1/2</sub>, half-life; and AUC<sub>0→∞</sub>, area under the curve of plasma concentration vs. time from t = 0 to t = ∞ after administration. Data represent mean ± SE (n = 3 or 4).

To be ca. 7-fold slower than that of nicotine. Elimination of cotinine from the blood was found to be 0.66 h<sup>-1</sup>; cotinine, 9.7 × 10<sup>-2</sup> h<sup>-1</sup>. The plasma concentration of cotinine exceeded that of nicotine in all tested periods, and the AUC<sub>0→∞</sub> values for nicotine and cotinine were calculated to be 103 ± 4.5 and 1,940 ± 88.1 ng h/mL, respectively. This was consistent with previous observations that absorbed nicotine was rapidly and extensively metabolized to inactive cotinine by CYP2A6 in human livers, which had a major impact on nicotine clearance.<sup>21</sup> The accumulation of cotinine might be associated with the adverse effects of cigarette smoking; however, cotinine has over two orders of magnitude lower affinity with nicotinic acetylcholine receptors than nicotine itself.<sup>22</sup>

Tissue distribution of nicotine and cotinine after subcutaneous administration of nicotine in rats: Furthermore, the tissue distributions of nicotine and cotinine at 0.5 and 2 h after subcutaneous administration of nicotine ditartrate solution (2 mg/kg) were examined using the developed UPLC/ESI-MS method (Fig. 2). In addition to the concentrations in cortex and bladder, where nicotinic acetylcholine receptors are expressed for neuronal control of detrusor function,<sup>14,23</sup> those in heart and salivary gland were also determined because cardiovascular disorders and dry mouth have been recognized as major adverse effects of nicotine. As shown in Figure 2A, the tissue concentrations of cotinine at 0.5 h after administration were found to be ca. 4- to 9-fold lower than those of nicotine. In particular, nicotine tended to accumulate in the bladder and salivary gland. At 2 h after administration, the tissue-to-plasma concentration ratios (K<sub>p</sub> values) of nicotine in the bladder, salivary gland, heart and cortex were estimated to be 57, 74, 9.2 and 21 mL/g tissue, respectively (Fig. 2B). In contrast, the K<sub>p</sub> values of cotinine in these tissues were calculated to be around 1 mL/g tissue, and much lower than this in heart. Thus, the tissue concentration of nicotine highly exceeded that of cotinine during the early period after administration, and the much lower blood concentration of nicotine might be responsible for the huge difference of K<sub>p</sub> values between nicotine and cotinine.

From these data, taken together with weak biological activities of cotinine, it is unlikely that nicotine intake or cigarette smoking would yield a cotinine concentration high enough to activate nicotinic receptors in the central and peripheral tissues, whereas cotinine seemed to be predominant in the blood. The deliverable nicotine in the central nervous system and bladder might be responsible for detrusor instability in smokers. However, further investigations such as pharmacodynamic characterization and analysis of receptor occupancy in each tissue are required to

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provide more reliable conclusions since cotinine could affect the affinity states of nicotinic receptors and thus counteract some of the effects of nicotinic agonists at the concentrations reached upon cigarette smoking, even without detectable stimulation.22

Urine concentration of nicotine and cotinine after subcutaneous administration of nicotine ditartrate (2.0 mg/kg body weight)

The mean Kp value (mL/g tissue) is indicated in each column. Data represent mean ± SE (n = 4). **P < 0.01; *P < 0.05, significant difference in nicotine concentration from the bladder.

**Fig. 2. Tissue distribution of nicotine and cotinine in rats at 0.5 h (A) and 2.0 h (B) after subcutaneous administration of nicotine ditartrate (2.0 mg/kg body weight)**

Nicotine and cotinine concentrations in various tissues after subcutaneous administration of nicotine ditartrate (2.0 mg/kg body weight).

In the present study, after single and repeated subcutaneous administration of nicotine ditartrate solution (2 mg/kg), urinary excretions of nicotine and cotinine were monitored using the UPLC/ESI-MS method (Fig. 3). Rapid and intense urinary excretion of nicotine was observed after subcutaneous administration of nicotine ditartrate (Fig. 3A), and the urine concentration of nicotine reached its maximal level (5.52 ± 0.58 µg/mL, corresponding to 34 µM) at 4 h after administration. Unlike the pharmacokinetic behavior in blood, the urine concentration of cotinine was much lower than that of nicotine up to ca. 6 h after administration, possibly due to the limited C-oxidation of nicotine in the bladder and/or low urinary excretion of cotinine. There was gradual elimination of both nicotine and cotinine in urine with elimination rates of 0.31 and 0.23 h⁻¹, respectively. Previously, our group showed that the IC₅₀ value of nicotine for brain [³H]nicotine binding was 17 nM, and Vainio demonstrated that the EC₅₀ for brain [³H]epibatidine binding was 300 nM for nicotine and 130,000 nM for cotinine. Thus, the IC₅₀ and EC₅₀ values for nicotinic receptor binding of nicotine were much lower than the urine concentration of nicotine in the current study, so nicotine in the urine might activate nicotinic receptors in the urinary bladder epithelium, thereby significantly affecting bladder function. Interestingly, when nicotine ditartrate (2 mg/kg) was
administered repeatedly (b.i.d., 10 days), nicotine was always predominant in urine, with the ratio of urinary excreted amount between nicotine and cotinine in the range 2.3–3.7 (Fig. 3B). Previously, Nakajima and co-workers demonstrated in real-time RT-PCR experiments on human tissues that mRNA for CYP2A6 was highly expressed in liver and breast and moderately expressed in lung. In contrast, the copy number of CYP2A6 mRNA in the bladder was found to be over two orders of magnitude lower than that in the liver. Although there might be some species differences in the expression level of CYP2A6 mRNA, it can be hypothesized that the metabolizing enzyme for nicotine, CYP2A6, in the bladder might be saturated by chronic treatment with nicotine.

Thus, pharmacokinetic studies using the UPLC/ESI-MS method demonstrated that single and chronic administration of nicotine resulted in a rapid and intense increase of urine nicotine, possibly producing phasic contraction of the urinary bladder directly. One mechanism to explain the association of current smoking with detrusor instability might be nicotine in urine. Masuda and co-workers demonstrated that intravesical injection of nicotine significantly decreased intercontraction intervals in a dose-dependent fashion and that the effects were abolished by the co-application of mecamylamine as well as by capsaicin pretreatment. These results suggest the induction of detrusor overactivity by nicotinic receptor activation in capsaicin-sensitive C-fiber afferents in the bladder. The up-regulation of nicotinic and muscarinic receptors in the bladder of rats repeatedly administered nicotine (our unpublished observation) may be involved in the development of detrusor instability following chronic smoking. Variability of nicotine metabolism could be an important determinant of nicotine clearance in both humans and rats, so that genetic polymorphisms of the CYP2A6 gene might lead to the variable frequency of voiding dysfunction in smokers. However, there might be species differences in nicotine metabolism; therefore, monitoring of nicotine and its metabolites in human urine after single and chronic administration of nicotine is necessary for further clarification of the association between smoking and voiding dysfunction.

Conclusions

In conclusion, a new UPLC/ESI-MS analytical method was developed for determination of nicotine and cotinine, and the method met commonly used validation criteria in terms of linearity, accuracy, recovery and robustness. The application of this method to pharmacokinetic studies in rats revealed that absorbed nicotine is rapidly and extensively metabolized into cotinine in blood; however, nicotine was predominant in cortex, bladder and urine. In particular, repeated administration of nicotine in rats resulted in ca. 3-fold higher accumulation of nicotine than that of cotinine in urine. These findings, taken together with the pharmacological characteristics of nicotine and cotinine, support the hypothesis that nicotine, rather than cotinine, plays an important role in detrusor instability in cigarette smokers.

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