Inter-Individual Variability of Cytochrome P450 2A6 Activity in Javanese Smokers’ Urine

Variasi Aktivitas Enzim Sitokrom P450 2A6 pada Individu Perokok Suku Jawa

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Abstract
Nicotine, the active compound on cigarette, was a compound that responsible to smoking addiction. The rate of nicotine metabolism is hypothesized to be a determinant of how much a person smokes. That is, rapid metabolizers would be expected to smoke more than slow metabolizers. Nicotine is metabolized extensively by the liver enzyme cytochrome P450 2A6, primarily to cotinine. Cotinine then metabolized by cytochrome P450 2A6 to 3'-hydroxycotinine. The ratio of metabolite to parent (i.e., 3OH-Cot/Cot) would be expected to reflect CYP2A6 activity. The ratio of 3OH-Cot/Cot in the 50 urine smokers was measured by HPLC method with an C8 fully endcapped residual silanol-type column coupled and UV detection with liquid-liquid extraction. All of the subject had been genotyped as CYP2A6*1/*4. Upon completion of the study, the CYP2A6 activity determined by ratio 3OH-Cot/Cot were between 0.01-0.93. There were 84% of subject identified as slow metabolizer and only 16% of subject identified as fast metabolizer. The ratio of 3OH-Cot/Cot was positively correlated with the number of cigarettes smoked per day (r = 0.327, p = 0.020). This finding supports the hypothesis that the rate of nicotine metabolism is a determinant of the level of cigarette consumption and supports the use of the 3OH-Cot/Cot ratio as a non-invasive marker of nicotine metabolism.

Keywords: nicotine; cotinine; trans-3'-Hydroxycotinine; Cytochrome P450 2A6
Introduction

Tobacco consumption kills at least 200,000 people annually in Indonesia, and tobacco use has serious negative health impacts for nearly every organ in the body. Although smoking has been clearly identified as a direct cause of multiple medical disorders, including cancers, cardiovascular, and respiratory diseases, people continue to smoke. Nearly all (97%) tobacco users in Indonesia smoke cigarettes. Smokers are predominantly male, although the prevalence of female smoking is increasing. Smoking prevalence is 34%, and 63% of men smoke.\(^1\) Cigarette consumption in Indonesia is number fourth in the world; after China, USA and Russia. Community Based Surveys, Global Adult Tobacco Survey (GATS) Indonesia 2011, shows significant increase of active male cigarette smokers in Indonesia, that is from 53.9% in 1995 to 67.0% in 2011. The smoker is more prevalent in male (67.0%) as compared to female (2.7%).\(^2\)

Nicotine is of importance as the addictive chemical in tobacco, pharmacotherapy for smoking cessation and a useful probe drug for phenotyping cytochrome P450 2A6 (CYP2A6).\(^3\) The plasma concentration of nicotine varies among tobacco users, due in part to differences in nicotine metabolism. An important route of nicotine metabolism involves cytochrome P450 (CYP) 2A6 / aldehyde oxidase (AO) - mediated conversion to cotinine (Cot). This is thought to be followed by further CYP2A6-mediated oxidation of Cot to trans-3´-hydroxycotinine (3OH-Cot)\(^4\) (Figure 1).

Since the conversion of Cot to 3OH-Cot entirely due to CYP2A6 activity, the ratio of 3OH-Cot to Cot (referred to as the nicotine metabolite ratio or NMR), provides a convenient measure to phenotype individuals for CYP2A6 activity and could serve as a marker for nicotine metabolism rate.\(^5\) This method is being used for large-scale pharmacogenetic studies. Metabolic activation of some carcinogenic nitrosamines, including some present in tobacco, is mediated by CYP2A6,\(^6–9\) and that is another reason for interest in this phenotypic marker.

Nicotine metabolism by cytochrome P450 2A6 (CYP2A6) varies across ethnicity/race and is hypothesized to affect smoking behavior.\(^10–12\) The NMR in the urine of young adult smokers was higher in whites and Hispanics than in blacks and Asians.\(^13\)

Cot and 3OH-Cot have relatively longer half-life than nicotine and it can be easily detected in urine, serum, plasma and saliva. The 3OH-Cot concentrations in urine generally exceed Cot concentrations by 3-4 fold, consequently, determination of 3OH-Cot, as well as Cot, might provide a more sensitive measure of exposure, especially when urine is used.\(^14–17\) In the present study, we describe a simple HPLC method using an C8 fully endcapped residual silanol-type column coupled with UV detection for determination of Cot and 3OH-Cot in human urine with liquid-liquid extraction. According to a previously published procedure, it was not easy to eluate Cot from HPLC in a short time.\(^18\) This method can easily resolve Cot from 3OH-Cot and acetanilide (Internal Standard) in a short period of 10 min and serves as a useful tool for the assessment of the 3OH-Cot/Cot ratio. Since CYP2A6 has been identified as the enzyme that responibles for the clearance of many drugs and environmental chemicals, in this study, the activity of CYP2A6 is investigated in Javanese Indonesian smoker. The objectives of this study were to determine the enzyme CYP2A6 activity in Indonesian smokers using the NMR as phenotype of these enzyme.

![Figure 1. Metabolism of Nicotine to Cotinine and Trans-3-Hydroxycotinine.](image-url)
Method

Cotinine and trans-3-hydroxycotinine were provided by Sigma Chemical Co., St. Louis and Santa Cruz Biotechnology Inc. Acetanilide as internal standard was an analytical grade obtained from Merck, Darmstadt Germany. HPLC grade methanol was; obtained from Fisher Scientific, UK., sodium hydroxide, chloroform and ethanol used were of analytical grade (J. T. Baker Chemical Co., Phillipsburg, NJ.), water purification system was used to obtain the purified water for the HPLC analysis.

The instruments used were:

a. **HPLC systems.** A Shimadzu (Kyoto, Japan) HPLC system was used consisting of a LC 2010HT system controller equipped with two LC-8A pumps, an UV detector (SPD-20A/20AV) (Shimadzu Corporation, Kyoto, Japan) and a high throughput autosampler injector. The detector was set at 260 nm at a sensitivity of 0.0001 AUFS.
b. **Columns.** Chromatographic separation was achieved with a Premier C8 fully endcapped residual silanol column (5µm, 4.6x250 mm, Shimadzu, Kyoto, Japan) at room temperature.
c. **Software.** The data were acquired and processed using LC solution (Version 1.03 SP3, Shimadzu Corporation, Kyoto, Japan) software running under Windows XP on a Pentium PC.

Chromatographic separation was achieved with a Premier C8 fully endcapped residual silanol column at room temperature (25°C). The mobile phase consisted of methanol and ammonium acetate 5 mM (50 : 50) at a flow rate of 1.0 ml/min. The samples were kept at room temperature (25°C) and a volume of 20 µl was injected for analysis. Ultraviolet detection was achieved with a SPD-20A/20AV UV-VIS detector at 260 nm at a sensitivity of 0.0001 AUFS. The total run time was 8 minutes.

To determine cotinine and trans-3 Hydroxycotinine in urine samples from male smokers, the study had been done on 2012 and had been approved by the Ethics Committees of Medical Research Gadjah Mada University (Yogyakarta, Indonesia). A total of 50 male healthy Javanese Indonesian smokers were recruited from students and staffs of Sanata Dharma University. They were asked to provide information on the numbers of cigarettes smoked daily (Table 1). The smokers were selected according to their smoking habits which were categorized into three levels: light smokers (CPD: 1-10), intermediate smokers (CPD: 11-20) and heavy smokers (CPD: 21-30). Smoking status was assigned based on questionnaires, which requested information on smoking history, such as the number of cigarettes per day (CPD), the nicotine content of the cigarettes, which they usually smoke, and time to the first cigarette of the day, which is generally accepted as a clinical index for nicotine dependence. The subject had smoked for minimum 1 year, not currently planning to stop smoking, between 18 and 50 years-old, took no concurrent medications, and had no illnesses requiring investigation or treatment. Urine samples were obtained from test individuals by collecting the first urine in the morning (5-15 mL) and were kept frozen at -20°C in polypropylene bottles until analysed (1 month).

Urinary Cot and 3OH-Cot were extracted and prepared for HPLC analysis as described by Patramurti et al. The samples of the first urine in the morning of 50 smokers were separately collected. Quantification of Cot and 3OH-Cot were done by plotting Cot and 3OH-Cot to internal standard (acetanilide) peak area ratio as a function of acetanilide concentration. The urinary NMR was determined using the formula:

$$\text{NMR} = \frac{\text{level (3OH - Cot)}}{\text{level (Cot)}}$$

The distribution of the NMR among subjects was tested for normality using Kolmogorov–Smirnov test. One Way Anova followed by Tukey multiple comparison test was used to determine differences in NMR by smoker groups. Pearson’s correlation analysis was also conducted to determine whether NMR was related to CPD.

Results

We previously reported simultaneous analysis of Cot and 3OH-Cot using HPLC-UV. According to these previously published method, Cot, 3OH-Cot, and Acetanilide in spiked
samples, were eluted rapidly with a complete resolution and sharp symmetrical peaks. The total chromatographic run time for nicotine biomarkers in meconium was 8 minutes. Resolution times of 3OH-Cot, Cot, and asetanilide as internal standard were 3.966, 4.658 and 6.967 minutes, respectively.

The calibration curves for 3OH-Cot and Cot were linear within the range from 1-5 µg/mL for 3OH-Cot and 2-10 µg/mL for Cot with correlation coefficients of 0.998 and 0.998. Relative recoveries for both 3OH-Cot and Cot were 82-93% for 3OH-Cot (at 1, 3 and 5 µg/mL) and between 82-98% for Cot (at 2, 6, and 10 µg/mL). Both the intra- and inter-day RSD were less than 10% over the range 1-5 µg/mL for 3OH-Cot and 2-10 µg/mL for Cot. The LOQ for the assay was 0.4 µg/mL for 3OH-Cot and 0.75 µg/mL for Cot with intra-day RSD of 11.14 and 11.52%, respectively, and inter-day RSD of 13.79 and 15.82%, respectively. The LOD was 0.1 µg/mL for 3OH-Cot and 0.2 µg/mL for Cot.

The liquid-liquid extraction HPLC-UV assay for the simultaneous detection of Cot, and 3OH-Cot concentrations in the urine Javanese Indonesian smokers was sensitive and reproducible; the between-day variations were within acceptable limits; and the assay remained linear in excess of the necessary ranges of detection. Therefore, the liquid-liquid extraction HPLC-UV assay can be used to identify and quantify levels of Cot as well as 3OH-Cot in the urine smokers.

The described HPLC method was applied to determine NMR urine samples of male smokers to describe CYP2A6 activity. Nicotine have been used as an in vivo probe for CYP2A6, based on their preferential catalysis of different steps in the metabolism of nicotine. Urine NMR is more biologically reasonable to measure CYP2A6 activity since it incorporates all 3OH-Cot generated from cotinine. In this study, using NMR, the distribution of CYP2A6 activity in 50 volunteers, aged 18 to 47, in the Javanese Indonesian was investigated. The 50 smokers recruited had an average age of 35 (SD=9) years, and an average age at which they had started to smoke regularly of 16 (SD=3.4) years. All of the subjects smoked filter cigarettes. A great inter-individual variation of CYP2A6 activity, determine by NMR in the 24 hour urine sample, was observed with a range from 0.10 to 0.93. The coefficient of variation of CYP2A6 activity in this population was 34.0%. The mean of CYP2A6 activities among three groups of smokers, measured by the urinary NMR, ranged from 0.28 to 0.45, and the distributions of NMR were normal (Table 1).

The mean CYP2A6 activities among subjects, measured by the urinary NMR, were found to be significantly higher among heavy smokers vs. light smokers (p value 0.015 < 0.05) and heavy smokers vs. intermediate smokers (p value 0.017 < 0.05).

No statistically significant differences in NMR were seen among light and intermediate smokers (p value 1.000 > 0.05).

Table 1. Demographic Characteristics of Subjects According to Smoking Habits and CYP2A6 Activity Measured by the Urinary NMR

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Smoking Habits</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Number of subjects</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>Age Mean ± SD</td>
<td>33.6 ± 9.1</td>
<td>34.94 ± 9.56</td>
</tr>
<tr>
<td>Range CPD</td>
<td>18-45</td>
<td>18-46</td>
</tr>
<tr>
<td>NMR Mean ± SD</td>
<td>8.15 ± 9.11</td>
<td>13.06 ± 1.03</td>
</tr>
<tr>
<td>Range</td>
<td>6-10</td>
<td>12-14</td>
</tr>
</tbody>
</table>

Characteristics: Number of subjects, Age Mean ± SD, CPD Mean ± SD, NMR Mean ± SD.
Discussion

This is the first study examining the relationship between CYP2A6 activity and smoking behaviors in Javanese Indonesian smokers. Inter-individual differences in the activity of drug metabolizing enzymes are of great importance in drug treatment. People with significant difference in drug metabolizing enzyme activity are at higher risk of drug toxicity or poor therapeutic outcome. Cytochrome P450 enzymes are especially important in this respect, as they are responsible for the most reactions of drug metabolism. As some important carcinogens such as aflatoxin B1 and nitosamines are metabolized by CYP2A6, the lower activity of CYP2A6 in Javanese population poses them to a lower risk of cancer incidence due to active metabolites of these compounds. This may also be important in the metabolism and half-life of its drug substrates such as halothane and valproic acid.

The NMR, derived from urinary measures of cotinine and 3-hydroxycotinine, has been shown previously to correlate with the amount of cigarette consumption during ad libitum smoking, suggesting that rates of nicotine metabolism and clearance, at least in part, affect the amount smoked. In the present study, we found a positive correlation between the urine NMR and CPD in adult smokers, which indicates that faster nicotine metabolism increases smoking rates (Figure 2). The correlation between NMR and CPD among the smokers was also significant, but of low predictive value ($r = 0.375$, $p = 0.02$). We speculated that the low of relation between the NMR and CPD is associated with the relatively low level of smoking (average CPD = 13) among our subjects, who may not have been as nicotine dependent. Social reasons and the social environment play a role in smoking uptake, smoking maintenance and smoking Cessation.

The disposition of Cot to 3OH-Cot, similarly to the metabolism of nicotine to Cot, is CYP2A6 mediated and has been shown to drive cigarette consumption for nicotine level self-regulation. Faster nicotine metabolism leads to smoking more cigarettes is that heavier smoking results in higher 3OH-Cot/Cot ratios because of induction of nicotine metabolism. That is, heavy smokers metabolize nicotine primarily by pathways other than C-oxidation to cotinine, resulting in attenuation of the relationship

![Figure 2. Relationship between Urinary 3OH-Cot/Cot Ratios and the CPD Smoked During the 24 Hours before Urine Sampling. Pearson Correlation=0.375; P=0.020.](image)
between 3OH-Cot/Cot ratios and the number of cigarettes smoked.

This finding supports the hypothesis that the rate of nicotine metabolism is associated with the level of cigarette consumption. Benowitz, et al.\textsuperscript{5} showed a correlation coefficient of 0.32 between the 3OH-Cot/Cot ratios and the CPD among smokers. This value is very similar to those determined in our study, i.e., 0.375 for Pearson Correlation. The NMR, derived from urinary measures of Cot and 3OH-Cot, in line with previously study,\textsuperscript{5,27,29} are correlate with CPD, suggesting that rates of nicotine metabolism and clearance, at least in part, affect the amount smoked. Thus, inter-individual variability in nicotine metabolism appears to be an important factor that limits the quantitative application of 3OH-Cot/Cot ratios as a phenotypic measure of CYP2A6 activity.

**Conclusion**

The HPLC method using an C8 fully end capped residual silano-type column coupled with UV detection used in this research showed an adequate method for determination of Cot and 3OH-Cot in human urine. Our findings support the hypothesis that inter-individually based differences in nicotine metabolism among adolescent smokers are associated with the phenotypic expression of CYP2A6. This study provides further evidence that the NMR can serve as a phenotype marker of CYP2A6 activity.

**Suggestions**

The findings of this study provide evidence that among these sample study are more likely to be slower nicotine metabolizers. However, to evaluate the inter-individuals differences in the CYP2A6 activity in relation to polymorphisms of these enzymes among Javanese Indonesian smokers and the real effect of CYP2A6 gene defect on addiction to tobacco smoke, further studies that more precisely address smoking addictive behavior, tobacco consumption and current smoke intake via measurement of valid other biomarkers such as CO-hemoglobin and cotinine will be studied in the future.

**Acknowledgment**

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