Analysis of Nicotine Metabolites in Three Ethnic Groups

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Abstract

Nicotine is the primary addictive agent in tobacco. Nicotine metabolism influences smoking intensity, and therefore disease risk. A key enzyme in nicotine metabolism is P450 2A6. Smokers with low P450 2A6 activity tend to smoke less than those with higher activity.

The goal of this thesis research was to explore the relationship between P450 2A6 metabolism and the nicotine metabolite profile of three ethnic groups that have different frequency of *CYP2A6* variant alleles. We also investigated the effect of P450 2A6 activity on the formation of 4-hydroxy-4-(3-pyridyl) butanoic acid (hydroxy acid) relative to the metabolites of other nicotine metabolite pathways. Nicotine is metabolized primarily by *CYP2A6* catalyzed 5'-oxidation to cotinine and trans-3'-hydroxycotinine. Two additional pathways generate nicotine-N-oxide, nicotine-N-glucuronide. In addition, nicotine is metabolized by 2'-oxidation which generates hydroxy acid. We were able to add cotinine-*N*-oxide to a well-established LC-MS/MS analysis by collecting the methanol wash and to analyze hydroxy acid by inserting a strata X-A SPE column prior to the MCX. This newly developed LC-MS/MS method allow us to analyze nicotine and seven metabolites from a single urine sample. Specially, we quantified the distribution of hydroxy acid and cotinine-

N-oxide which are measured for the first time in the three ethnic groups and determined their formation did not rely on P450 2A6 activity.

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CHAPTER 1. BACKGROUND

1.1 Tobacco use and health consequences

Extensive research has been conducted on tobacco and its effects on human health. Studies have shown that smoking is associated with lung cancer, stomach cancer, liver cancer, pancreatic cancer, kidney cancer oropharyngeal cancer, cervical cancer, laryngeal cancer, esophageal cancer, ureter cancer, bladder cancer, colorectal cancer, and acute myeloid leukemia [1]. And smoking has a causal relationship with the respiratory system, immune system, cardiovascular system, respiratory digestive system, and reproductive system [2]. It is imperative to prevent or treat addiction to tobacco products.

Nicotine metabolism influences smoking intensity, and therefore disease risk. A key enzyme in nicotine metabolism is P450 2A6 (discussed more below) Smokers with low P450 2A6 activity tend to smoke less than those with higher activity and therefore decrease their carcinogen exposure [3]. P450 activity is significantly associated with lung cancer risk [4].

1.2 Nicotine and Nicotine metabolism

Tobacco smoke is made up of more than 5000 chemicals. The mixture contains many known or suspected human carcinogens and toxic substances, including over 70 known carcinogens [5]. The way to minimize exposure to these harmful compounds is to stop smoking. However, due to the intensity of nicotine addiction, many smokers find it difficult to quit smoking. Nicotine is not a carcinogen, but it can be said that it is the compound in tobacco that has the greatest impact on smokers' cancer risk. Nicotine sustains tobacco addiction and keeps people smoking [6]. Nicotine is the main tobacco alkaloid, accounting for 95% of the total alkaloid content, and its content in cigarettes is about 1.5% [7].

After inhalation, nicotine enters the circulation through the lungs. According to previous studies, nearly 90% of inhaled nicotine will be absorbed by smokers, and non-smokers can absorb nicotine from environmental smoke with an efficiency of about 70% [8]. It then enters the brain after seconds, where it easily diffuses into the tissues and selectively binds to nicotinic cholinergic receptors. This leads to the release of dopamine, thereby regulating the pleasurable experience of smoking. The research of compounds

that cause or reduce the damaging effects of tobacco or affect addiction is particularly important.

In the late 1950s, the study of nicotine metabolism was first started. Cotinine was identified as the main nicotine metabolite in the urine of smokers [9]. Benowitz et al. (1994) found that about 75% of nicotine absorbed by most smokers was converted to cotinine [10]. P450 2A6 was identified as the main human enzyme responsible for the 5'-oxidation of nicotine to cotinine [11]. About 4 to 7% of nicotine is converted to nicotine-*N*-oxide by oxidation of the pyrrolidine ring. Flavin monooxygenase (FMO) catalyzes the N-oxidation of the pyrrolidine ring [12]. In addition, about 3 to 5% of nicotine produce nicotine glucuronide by uridine diphosphate-glucuronosyltransferase (UGT) enzyme [13]. About 1% of nicotine is converted to nicotine isomethonium ion by methylation of the pyridine nitrogen [12]. Less than 1% of nicotine undergoes oxidative N-demethylation to become nornicotine [14].

In 2000, our laboratory confirmed the presence of a new pathway of nicotine metabolism [15]. Nicotine was shown to be metabolized to 4-(methylamino)-1-(3-pyridyl)-1-butanone by 2′-oxidation of nicotine. It is further metabolized to produce 4-oxo-4-(3-pyridyl) butanoic acid (keto acid) and 4-hydroxy-4-(3-pyridyl) butanoic acid (hydroxy acid). It is estimated that 10 to 15% of

nicotine is converted to keto acid and hydroxy acid in human [16]. The metabolic pathways of nicotine that have been characterize are in Fig.1. Efficient assays exist for most metabolites illustrated (red in Fig.1). One of the main purposes of my research is to develop a high-efficiency method to quantify hydroxy acid and three other nicotine metabolites (blue in Fig.1).

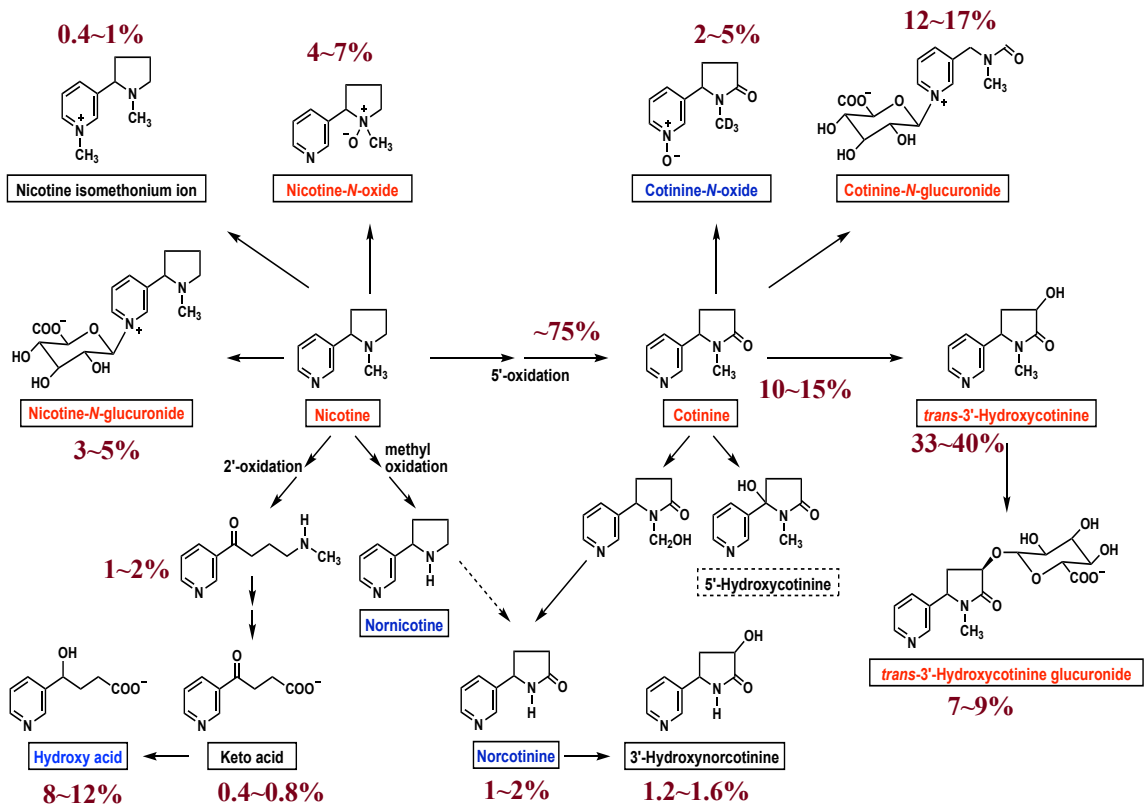

Figure 1. Nicotine metabolism pathways in white smokers.
The compounds with boxed names have been quantified as urinary metabolites in smokers [3]. Our laboratory has efficient methods to quantify metabolites in red. My research goal is to develop methods to quantify metabolites in blue.

1.3 Cotinine metabolism

In most smokers, three-quarters of nicotine is metabolized to cotinine and most of the cotinine continues to be further metabolized. *Trans* 3'-hydroxycotinine was characterized and found to be the main urinary cotinine metabolite and excreted in part as *trans* 3'-hydroxycotinine glucuronide [17]. P450 2A6 which catalyzes the 5′-oxidation of nicotine also catalyzes the metabolism of cotinine to 3′-hydroxycotinine. Cotinine glucuronide was identified and determined to have a slightly higher concentration than cotinine in the urine of smokers [18]. N-oxidation of cotinine occurs on the pyrrolidine nitrogen not the pyridine nitrogen as nicotine-*N*-oxidation, and the catalyst of this reaction is a P450 enzyme [12]. Cotinine-*N*-oxide has been detected in smokers' urine (about 2% to 5% of nicotine metabolites) [19]. In addition to the main pathways of 3'-oxidation, N-glucuronidation and N-oxidation, the minor metabolites of cotinine include norcotinine and 5′-hydroxycotinine. In our previous work, P450 2A6 was reported to catalyze formation of norcotinine and 5′-hydroxycotinine from cotinine *in vitro* [20]. Norcotinine was reported to account for 1% of nicotine metabolites in the smokers' urine [21]. Studies have shown that norcotinine formation can be from the demethylation of cotinine or the oxidation of nornicotine [22, 23].

The sum of the urinary concentration of nicotine and six metabolites (nicotine glucuronide, cotinine, cotinine glucuronide, 3'-hydroxycotinine, 3'-hydroxycotinine glucuronide, nicotine-N-oxide) is referred to as total nicotine equivalents (TNE). TNE is a good measure of total nicotine exposure. In the work presented here we will include four additional metabolites in the TNE measurement.

1.4 P450 2A6 (*CYP2A6*)

It can be seen from the previous description that P450 2A6 plays an important role in nicotine metabolism and catalyzes multiple oxidation reactions. P450 enzymes are heme monoxidases that can catalyze a variety of reactions. Cytochrome P450 enzymes are mainly membrane-associated proteins present in most tissues of the body, mainly in the liver. These enzymes play an essential role in the synthesis of hormones, cholesterol and steroids, and also have the function of metabolizing potentially toxic compounds, including drugs. The human *CYP2A* genes that encode P450 2A enzymes are located in a gene cluster on chromosome 19. *CYP2A6*, *CYP2A7*, and *CYP2A13* comprise the human *CYP2A* subfamily [24].

The *CYP2A6* gene is highly polymorphic with more than 75 variant alleles identified (https://www.pharmvar.org/htdocs/archive/cyp2a6.htm). This is reflected in the observed considerable interindividual variation in P450 2A6 activity that is observed both *in vitro* and *in vivo* studies. Three variant alleles that illustrate this are *CYP2A6*4, CYP2A6*9* and *CYP2A6*12. CYP2A6*4* is composed of homologous unequal crossovers with *CYP2A7* at several positions (*CYP2A6*4A-F*), which results in the deletion of the entire gene. *CYP2A6*9* contains a point mutation in the TATA box located in the promoter of the gene. *CYP2A6*12* originated from the unequal crossover between *CYP2A6* and *CYP2A7* [25]. Their P450 2A6 activity is lost or pretty low [26]. The relative frequency of *P450 2A6* gene variants is significantly different among ethnic groups [27].

The quantitative data analysis in Fig.1 is from white smokers who do not lack P450 2A6 activity. Nicotine metabolism pathway profiles of other ethnic groups are different as shown in Fig.2 [28]. Therefore, deepening the understanding of the different metabolism caused by P450 2A6 activity in smokers may help establish a personalized system for the detection, prevention and treatment of people at higher risk of smoking in order to avoid diseases related to smoking.

The metabolism of cotinine to 3′-hydroxycotinine is almost completely mediated by the P450 2A6 enzyme. In addition, the long half-life of cotinine and the dependence of cotinine on 3′-hydroxycotinine cause the relative levels of these metabolites to remain stable in smokers' plasma. Due to these reasons, the ratio of 3′-hydroxycotinine to cotinine in plasma has been characterized as a measure of P450 2A6 activity in smokers and is referred to as the nicotine metabolism ratio (NMR) [12, 29, 30].

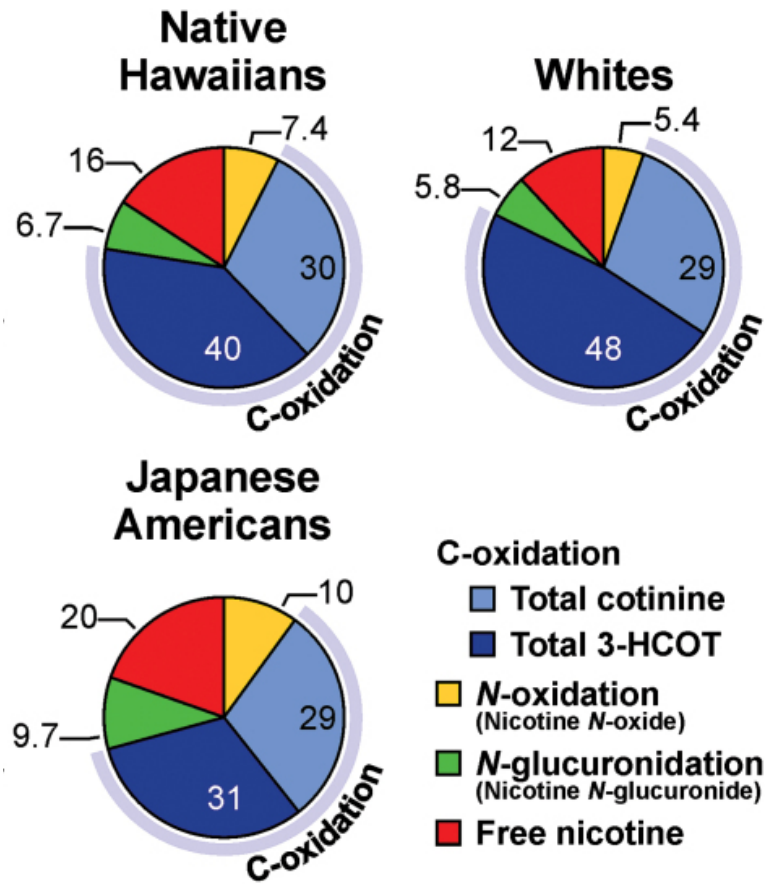

Figure 2. Proportion of nicotine metabolites in different ethnic groups. The values are the molar percent of nicotine and six metabolites excreted in urine. Total cotinine and total 3-HCOT(3′-hydroxycotinine) include both the free analyte and its glucuronide conjugate. Each slice of the pie is the mean percentage of the compound relative to TNE. From Murphy et al, Chem Res Toxicol. 2017 Jan 17;30(1): 410-419.

1.5 Goals of this research

This study will determine the relationship between P450 2A6 metabolism and the nicotine metabolite profile of three ethnic groups that have different frequency of *CYP2A6* variant alleles. In this project we will also investigate the effect of P450 2A6 activity on the formation of hydroxy acid relative to the metabolites of other nicotine metabolite pathways. The hypothesis is that if hydroxy acid is a downstream product of P450 2A6 metabolism and its concentration in urine will be significantly lower in smokers with little or no P450 2A6 activity. If there is no effect of P450 2A6 activity on the formation of hydroxy acid, a different P450 enzyme is likely contributing. To carry out this study we will develop the methods to analyze four nicotine metabolites (nornicotine, norcotinine, cotinine-N-oxide and hydroxy acid) that are not routinely quantified in smokers.

CHAPTER 2. METHODOLOGY

2.1 Human urine samples

Potential subjects were recruited through the University of Hawaii Cancer Center (UHCC). Recruitment criteria include 20 years of age or older; one of three target groups:

1. Japanese Americans-Two Japanese parents (or at least 3 Japanese grandparents)

2. White non-Hispanic-two parents of white non-Hispanic descent (or at least 3 grandparents of white non-Hispanic descent)

3. Native Hawaiian will include individuals with at least one parent of Hawaiian descent

; Smoked at least 5 cigarettes a day for the past three months.

81 Japanese Americans, 107 White non-Hispanic and 104 Native Hawaiian were included in our study.

The subjects collected their first morning urine sample for biomarker analysis. The sample was placed in a biohazard bag with an ice pack to keep it cool

until aliquoted into a 4.5 ml cryovial. The vial was barcoded for identification and stored at −20°C.

2.2 Chemical and Reagents

[d_3-Methyl]-nicotine, [d_3-methyl]-cotinine, [d_3-methyl]-3′-hydroxycotinine, [d_3-pyridyl]-nicotine-*N*-oxide, [d_3-methyl]-cotinine-*N*-oxide, [d_4-pyridyl]-Nornicotine, [d_4-pyridyl]-Norcotinine and [(4,3,3)-d_3]-4-hydroxy-4-(3-pyridyl) butanoic acid was purchased from Toronto Research Chemicals. All other solvents and reagents were of highest analytical grade and purchased from Sigma or Thermo Fisher Scientific.

Working solutions of the internal standard (IS) were prepared in water and contained 0.0485 ng/µl d_3-nicotine, 0.01228 ng/µl d_3-cotinine, 0.1773 ng/µl d_3-3′-hydroxycotinine, 0.0114 ng/µl d_3-nicotine-*N*-oxide, 0.1 ng/µl d_3-cotinine-*N*-oxide, 0.02 ng/µl d_4-nornicotine, 0.02 ng/µl d_4-norcotinine.

2.3 Analysis of nicotine and its 6 metabolites (Nornicotine, Nicotine, Nicotine N-oxide, 3′-hydroxycotinine, norcotinine and cotinine)

2.3.1 SPE Method

Urine samples were analyzed with or without prior treatment with β-glucuronidase to release glucuronic acid from nicotine, cotinine and 3′-hydroxycotinine [27]. If β-glucuronidase treated samples were analyzed, the nicotine, cotinine and 3′-hydroxycotinine quantified are referred as total. The urine sample was diluted 10 times and mixed thoroughly with the prepared internal standard, then they were loaded to SPE with an Oasis MCX 96-well Plate (10 mg, 30 μm). There are 4 high positive controls, 2 low positive controls and 10 water blanks in each plate. The plate was preconditioned with 2% NH_4OH in methanol (400 μl) and H_2O (400 μl) followed by 0.5% formic acid in water (400 μl). The column was washed with 0.5% formic acid in water (200 μl) and methanol (400 μl). The methanol washing eluant was collected. The methanol washing eluant was dried under nitrogen and stored at -20°*C* prior to analysis for cotinine-N-oxide. All other metabolites were eluted from the column with 2% NH_4OH in methanol (200 μl). Before eluting, 50 μl of 10% formic acid in methanol was added to each well of the collection plate to prevent nicotine from evaporating. The eluents were dried under nitrogen and the plates were stored at -20°*C*.

2.3.2 LC-MS/MS Method

Remove the dry plate from the freezer and allow to warm to room temperature before resuspending with 25 µl of 100 mM ammonium acetate in methanol. Analysis was performed on a TSQ Vantage triple-quadrupole mass spectrometer (Thermo Scientific, Waltham, MA) interfaced with a Dionex Ultimate 3000 UHPLC system (NCS-3500RS pump and WPS-3000PL autosampler).

Analysis of nicotine, nornicotine and nicotine-*N*-oxide was performed on a Waters Atlantis HILIC column, 5 µm, (300 µm x 100 mm) (part # 186008975). Sample injection volume was 3 µL. The mobile phases consisted of 0.1% TFA in water (A) and 0.5% formic acid in acetonitrile (B). Nicotine, nornicotine and nicotine-*N*-oxide were eluted with 91% B and 9% A at a flow rate of 23 µL/min. The column was operated at 40°C. MS acquire time is 4.5 min.

After analysis of the nicotine, nornicotine and nicotine-*N*-oxide, the plates were dried again and resuspended with 30 µl of 20 mM ammonium acetate in water for the second injection to analyze nicotine, nornicotine and nicotine-*N*-oxide.

Analysis of cotinine, norcotinine and 3′-hydroxycotinine were performed on a Luna C18 column, 3 µm, (150 x 0.5 mm) (part # 00F-4251-AF). Sample injection volume was 3 µL. The mobile phases consisted of 20mM ammonium acetate (A) and acetonitrile (B). Cotinine, norcotinine and 3′-hydroxycotinine were eluted with a linear gradient from 7 to 20% B over a period of 5 min and holding at this composition for 3 min. The gradient was then returned to 7% B. The flow rate was 10 µL/min. The column was operated at 40°C. MS acquire time is 10 min.

The dry MCX-methanol fraction was resuspended in 30 µl of 10 mM ammonium acetate in water. Analysis of cotinine-*N*-oxide was performed on a Luna C18 column, 3 µm, (150 x 0.5 mm) (part # 00F-4251-AF). Sample injection volume was 3 µL. The mobile phases consisted of 10mM ammonium acetate (A) and acetonitrile (B). Cotinine-*N*-oxide was eluted with 4% B and 96% A at a flow rate of 10 µL/min. The column was operated at 40°C. MS acquire time is 10 min.

For all three LC-MS/MS analyses, the Electrospray Ionization (ESI) source was operated in positive ion mode at ambient temperature. The ion source parameters were set as follows: spray voltage 4000 V, capillary temperature 300 °C, sheath gas pressure 20. The quadrupoles Q1 and Q3 were operated at

an isolation width of 0.4 Da and 0.7 Da. Scan width was set at 0.3 *m/z* with a scan time of 0.05 s. The mass transitions monitored were as follows: 1st LC−MS/MS analysis: nornicotine, *m/z* 149.05→130.05 and *m/z* 149.05→117.05; d_4-nornicotine, *m/z* 153.05→134.05 and *m/z* 153.05→121.05 (collision energy 24 V for *m/z*,130.05, *m/z* 134.05, *m/z*, 117.05 and *m/z*, 121.05); nicotine, *m/z* 163.05→130.05 and *m/z* 163.05→117.05; d_3-nicotine, *m/z* 163.05→130.05 and *m/z* 163.05→117.05 (collision energy 24 V for m/z,130.05 and m/z 117.05, m/z); nicotine-N-oxide, *m/z* 179.07→130.05 and *m/z* 179.07→117.05; d_3-nicotine-N-oxide, *m/z* 182.07→130.05 and *m/z* 182.07→117.05 (collision energy 28V for m/z,117.05, 21V for 130.05). 2nd LC−MS/MS analysis: norcotinine, *m/z* 163.12→80.06, 163.12→118.15 and *m/z* 163.2→135.14; d_4-Norcotinine, *m/z* 167.12→84.20, 167.12→121.14 and *m/z* 167.2→139.15 (collision energy 25V for *m/z*, 80.06 and *m/z*, 84.20 , 20 V for *m/z*, 118.15 , *m/z*, 121.14 *m/z*, 135.14 and *m/z*, 139.15); cotinine, *m/z* 177.06→80.06 and *m/z* 177.06→98.06; d_4-cotinine, *m/z* 180.06→80.06 and *m/z* 180.06→101.06 (collision energy 20 V for *m/z*, 80.06, *m/z*, 98.06 and *m/z*, 101.06); 3′-hydroxycotinine, *m/z* 193.13→80.06 and *m/z* 193.13→134.06; d_3-3′-hydroxycotinine, *m/z* 196.13→80.06 and *m/z* 196.13→134.06 (collision energy 22V for *m/z*, 80.06 and 16V for *m/z*, 134.06). 3rd LC−MS/MS analysis:

Cotinine-N-oxide, *m/z* 193.2→96.14, 193.2→98.14 and *m/z* 193.2→162.14; d_3-cotinine-N-oxide, *m/z* 196.2→96.14, 196.2→101.14 and *m/z* 196.2→162.14 (collision energy 23 V for *m/z*, 96.14, *m/z*, 98.14 and *m/z*, 101.14, 17 V for *m/z*, 162.14); Concentrations of the metabolites were calculated from the ratio of the peak area obtained by selected reaction monitoring of the mass transitions for the analyte and its deuterated internal standard.

2.4 Hydroxy acid (HA) Analysis

2.4.1 SPE Method

The 10 µl urine samples were diluted 10 fold and mixed thoroughly with the prepared 25 µl internal standard (0.136 ng/µl d_3-hydroxy acid) and 300 µl 25mM ammonium acetate pH 8.5. Then they were loaded to Strata-X 33 µm Polymeric Reversed Phase columns (30 mg / 3 mL). Each column needs to be preconditioned with methanol (1000 µl), water (500 µl) and 25mM ammonium acetate pH 8.5(500 µl). The column was washed with water (1000 µl) and methanol (1000 µl). Hydroxy acid was eluted from the column with 5% formic acid in methanol (400 µl). The eluents were dried under nitrogen and stored at -20°C before resuspended in 10 mM Ammonium acetate in water (60 µl).

2.4.2 LC-MS/MS Method

Analysis of hydroxy acid was performed on a Zorbax C18 column, 5µm, (250 x 0.5mm). Sample injection volume was 3 µL. The mobile phases consisted of 5mM ammonium acetate (A) and acetonitrile (B). Hydroxy acid was eluted with a linear gradient from 3 to 25% B from 4.5 min to 5.5 min and holding at this composition for 3.5 min. The gradient was then returned to 3% B. The flow rate was 15 µL/min. The column was operated at 40°C. MS acquire time is 15 min.

The Electrospray Ionization (ESI) source was operated in positive ion mode at ambient temperature. The ion source parameters were set as follows: spray voltage 3000 V, sheath gas pressure 10. The peak width of Q1 and Q3 were operated at 0.4 and 0.7. Scan width was set at 0.3 *m/z* with a scan time of 0.05 s. The hydroxy acid mass transitions monitored were *m/z* 182.07→118.00 and *m/z* 182.07→108.00; d_3-hydroxy acid, *m/z* 185.10→120.00 and *m/z* 185.10→109.00 (collision energy 21V for *m/z*, 118.00, *m/z*, 120.00, 32V for *m/z*, 108.00 and *m/z*, 109.00).

2.5 Statistics

The glucuronide conjugates were calculated from the difference between total and free analytes. Total nicotine equivalents (TNE) were calculated as the sum of total nicotine, total cotinine, total 3′-hydroxycotinine, nornicotine, nicotine-*N*-oxide, norcotinine, cotinine-*N*-oxide and hydroxy acid. The LOQ for nicotine, cotinine, 3′-hydroxycotinine, nicotine-*N*-oxide, cotinine-*N*-oxide, nornicotine, and norcotinine (10 µl urine) were 30.2 ng/ml, 3.5 ng/ml, 11.3 ng/ml, 13.6 ng/ml, 10.6 ng/ml, 9.6 ng/ml and 1.1 ng/ml respectively. The LOQ for hydroxy acid is 0.05 nmol/mL. LOQ was determined as 3-times the blank for each analyte.

CHAPTER 3. RESULTS

3.1 Method development

3.1.1 Quantitation of Nicotine, Nornicotine, Nicotine-N-oxide and 3′-hydroxycotinine, Norcotinine, Cotinine

The quantitation method used is modified to include nornicotine and norcotinine from a previously developed method. Our lab has developed this sensitive LC-MS/MS assay for the quantification of nicotine and its metabolites [28]. After solid phase extraction, HPLC separations were carried out on a capillary hydrophilic interaction chromatography column (HILIC) and a reversed phase chromatography column (Luna C18). The analytes were monitored by tandem mass spectrometry and electrospray with positive ionization. The detailed parameters are in the method section. The amount of nicotine, cotinine and their metabolites are quantified based on the use of known amount of deuterated internal standards (d_3 or d_4). The modified method added two additional internal standards d_4-nornicotine and d_4-norcotinine.

To quantify nicotine and 5 metabolites, we collected data using single reaction monitoring (SRM). For each metabolite, two transitions were monitored. The ratio between the two monitored transitions is used to confirm that we are only

measuring target analyte and not impurities with similar quality transitions. For nornicotine, nicotine, nicotine-*N*-oxide, we used *m/z* 149→ 130, *m/z* 163→ 130, and *m/z* 179→ 130 transitions to quantify, and *m/z* 149→ 117, *m/z* 163→ 117, and *m/z* 179→ 117 as qualifier ions. For 3′-hydroxycotinine, norcotinine, cotinine, we used we use *m/z* 193→ 134, *m/z* 163→ 80, and *m/z* 177→ 98 transitions for quantification, and *m/z* 193→ 80, *m/z* 163→ 135 and *m/z* 177→ 80 as qualifier ions.

An example of the LC-MS/MS analysis is shown in Fig.3. In this sample, the analyte peak is presented on the left and the internal standard is on the right for each compound. The concentration of its nornicotine is 230 ng/ml, nicotine is 2550 ng/ml. Nicotine-*N*-oxide is 3020 ng/ml. 3′-hydroxycotinine 5120 ng/ml, norcotinine 260 ng/ml, cotinine 1830 ng/ml.

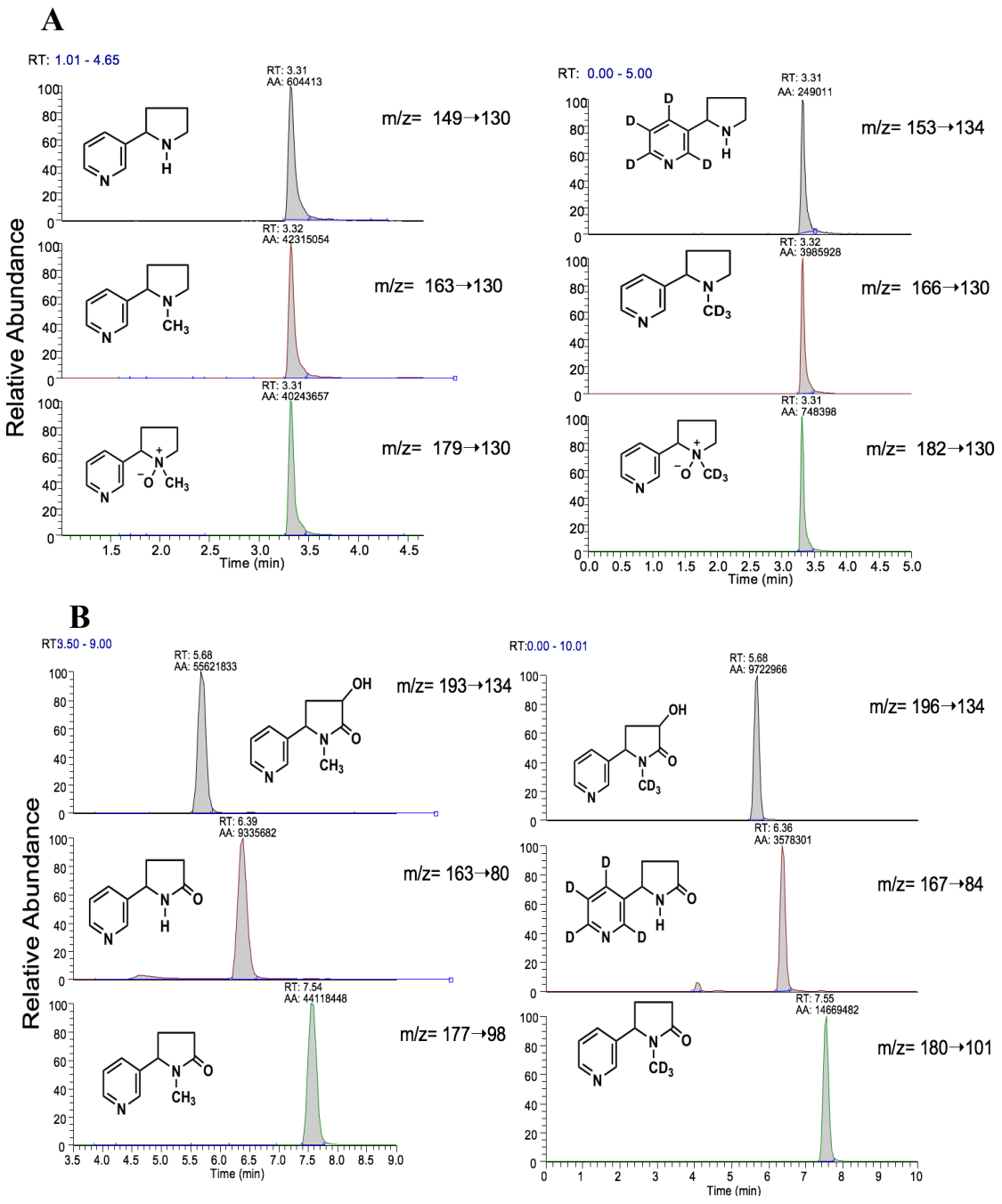

Figure 3. LC-MS/MS SRM analysis of a smoker's sample
for (A) nornicotine, nicotine and nicotine-*N*-oxide on a HILIC column, and
for (B) 3′-hydroxycotinine, norcotinine and cotinine on a Luna C18 column.

3.1.2 Quantitation of Cotinine-N-oxide

Cotinine-*N*-oxide is formed by oxidation of the pyridine of cotinine. Unlike the other metabolites mentioned above, the nitrogen on the pyridine ring is oxidized, and cannot be protonated in acid. Therefore, it has no affinity for the MCX column (a cation exchange column). We assumed that it would be eluted during the column wash phase. To confirm this, a standard cotinine-*N*-oxide solution was added to the column, the methanol wash was collected, and the same amount d_3-cotinine-*N*-oxide was added to the collected wash. In duplicate samples, the ratio of d_0-cotinine-*N*-oxide to d_3-cotinine-*N*-oxide was used to calculate the recovery of cotinine-*N*-oxide; 41% of cotinine-*N*-oxide was eluted.

Cotinine-*N*-oxide generates a number of daughter ions. The major fragment ions are *m/z* 79.26, 96.19, 97.96, 98.44, 161.94, 192.99 as shown in Fig.4. Both d_0-cotinine-*N*-oxide and d_3-cotinine-*N*-oxide fragment to 96.19 as their major ion. This is because the fragmentation loses the pyrrolidine ring which contains the D_3. However, we were unable to use the *m/z* 193→ 96 transition to quantify cotinine-*N*-oxide in smokers' urine due to a significant amount of a co-eluted compound. Through experiments with standards and urine from smokers, we found these two fragments (98 for quantification and 162 for

control) gave reproducible quantitation of cotinine-*N*-oxide. Ionization conditions were adjusted to maximize the *m/z* 193→ 96 fragmentation.

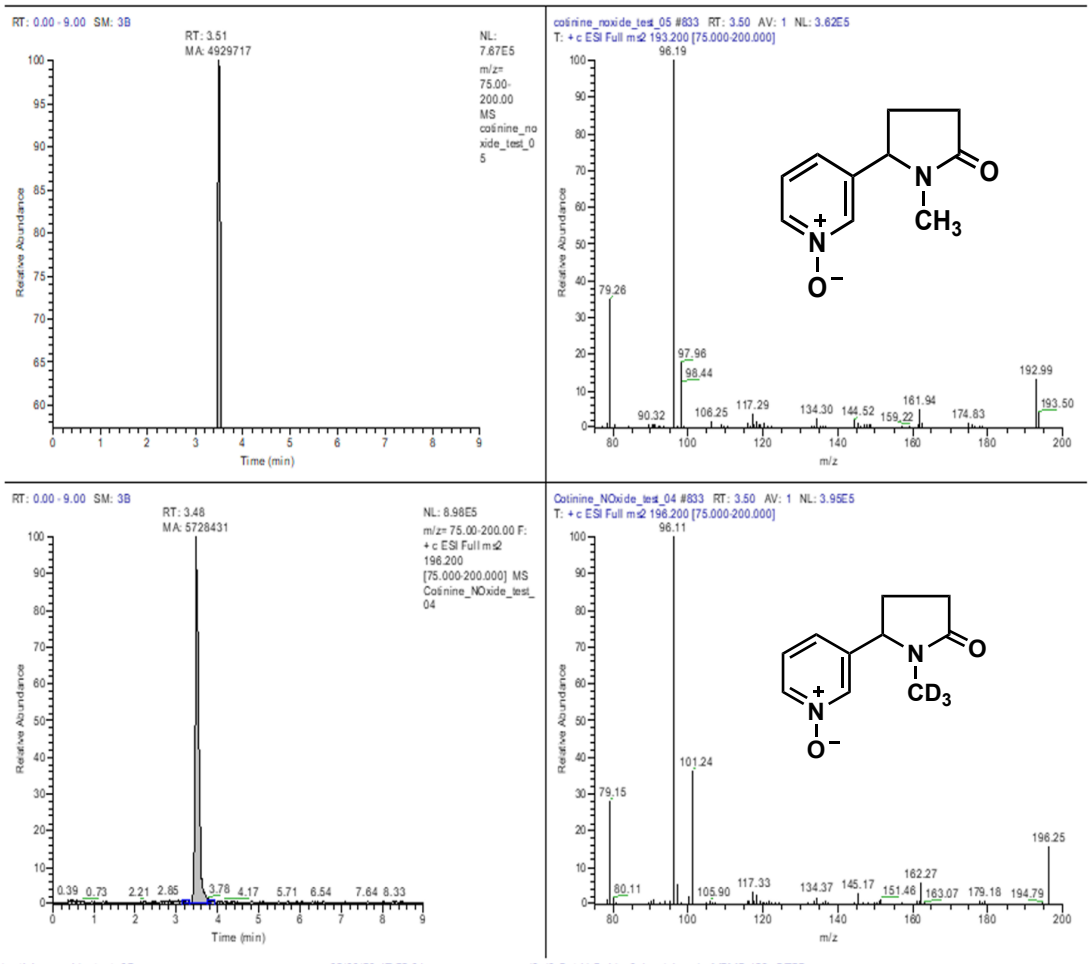

Figure 4. Liquid chromatography tandem mass spectrometry analysis of d_0-cotinine-*N*-oxide and d_3-cotinine-*N*-oxide.
(A) LC-MS of d_0-cotinine-*N*-oxide with monitoring ions of *m/z* 193. (B) MS/MS of d_0-cotinine-*N*-oxide, 3.51min peak. (C) LC-MS of d_3-cotinine-*N*-oxide with monitoring ions of *m/z* 196. (D) MS/MS of d_3-cotinine-*N*-oxide, 3.48min peak.

3.1.3 Quantitation of Hydroxy acid

Since hydroxy acid is a weak acid which is negatively charged at neutral pH, we used a strong anion-exchange column to separate hydroxy acid from other nicotine metabolites. We would like to find a column that retains hydroxy acid and lets all other nicotine metabolites wash through.

We set up two sets of samples and added the same amount of hydroxy acid before or after the strata X-A (strong anion exchange column). The ratio of their peak area shows that the recovery of hydroxy acid is 42.5%. In our preliminary experiment, urine samples were first adsorbed and eluted by the strata X-A, and it was found that the 7 nicotine metabolites mentioned above could not be detected in the eluate. Therefore, these metabolites are “lost” in the column wash but could be quantified by analyzing the strata X-A flow-through and column wash using the previously described MCX-LC-MS/MS protocol. Through experiments with standard samples containing d_0-hydroxy acid and d_3-hydroxy acid, we found the transitions: 182→ 108 for quantification, and 182→ 118 for qualification gave reproducible quantitation of hydroxy acid.

3.1.4 Method summary

Since we were able to add cotinine-*N*-oxide to the MCX workup and to insert a strata X-A column prior to MCX, we established a LC-MS/MS method to analyze nicotine and seven metabolites from one urine sample. The entire flowchart is shown in Fig.5. Four LC-MS/MS analysis methods were carried out for each sample to quantify nornicotine, nicotine, nicotine-*N*-oxide, 3′-hydroxycotinine, norcotinine, cotinine, cotinine -*N*-oxide and hydroxy acid.

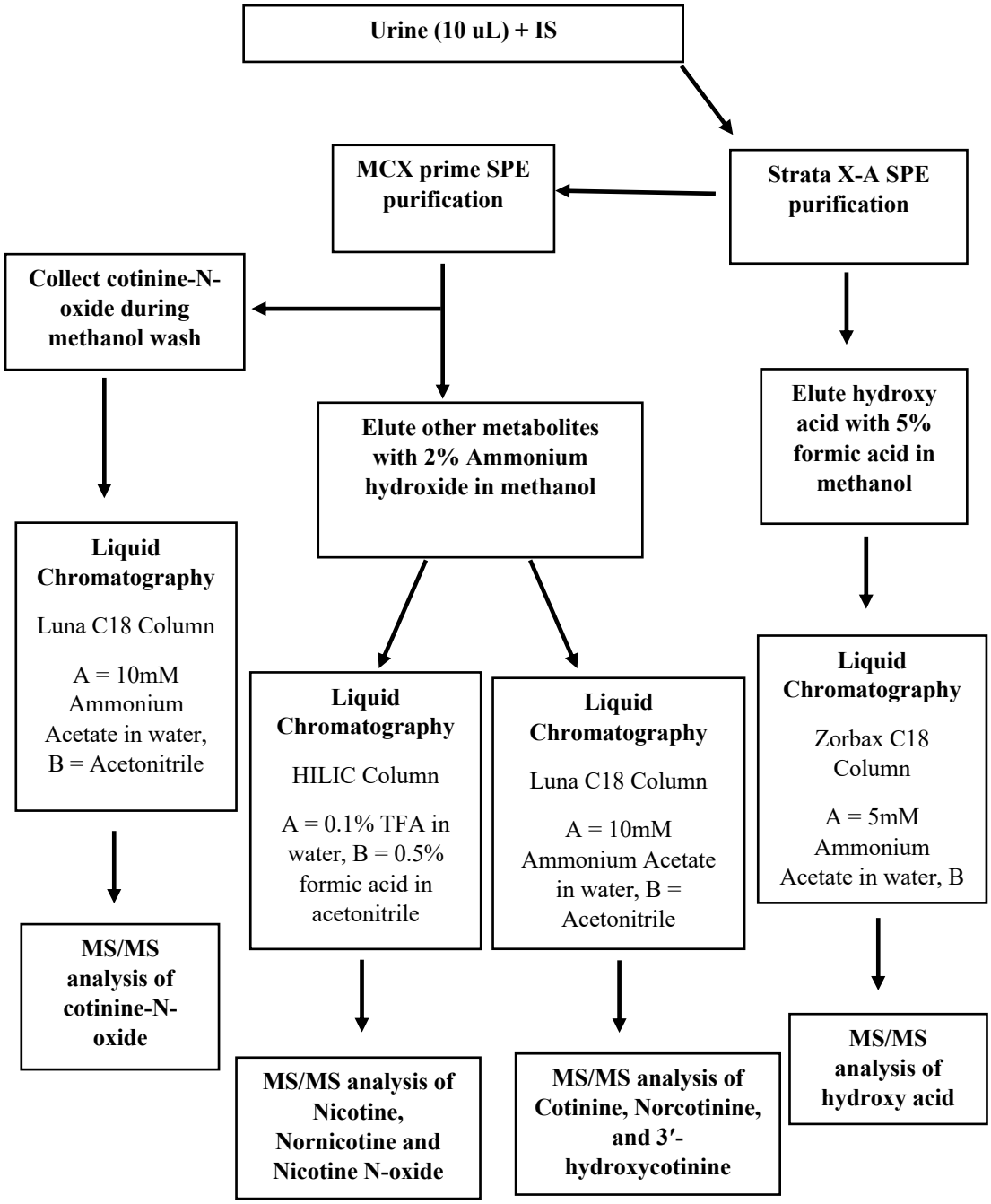

Figure 5. Outline of the LC-MS/MS method for the analysis of total nicotine metabolites in smokers.

3.2 Factors affecting nicotine metabolism

3.2.1 The difference in metabolites among the people of different ethnicity

Our previous research has shown that the pathway of nicotine metabolism varies by race [28]. Among different racial groups, Japanese excreted the lowest levels of cotinine and 3′-hydroxycotinine and the greatest amount of unchanged nicotine per nicotine dose as measured by TNE. The extent of nicotine C-oxidation by Native Hawaiian was less than White but greater than Japanese. My experimental data as shown in Fig.6 confirmed this result. There is a significant difference between the percentage of TNE excreted as 3′-hydroxycotinine of Japanese and that of White. On the other hand, our new method quantifies cotinine-*N*-oxide and hydroxy acid for the first time. We found that the concentration of these metabolites was not significantly different in different ethnic groups. In previous experiments, P450 2A6 *in vitro* nicotine metabolism resulted in the formation of hydroxy acid [31]. Cotinine-*N*-oxide was a minor product of P450 2A6 catalyzed cotinine metabolism [32]. Therefore, we want to further study the relationship between P450 2A6 activity and these two metabolites.

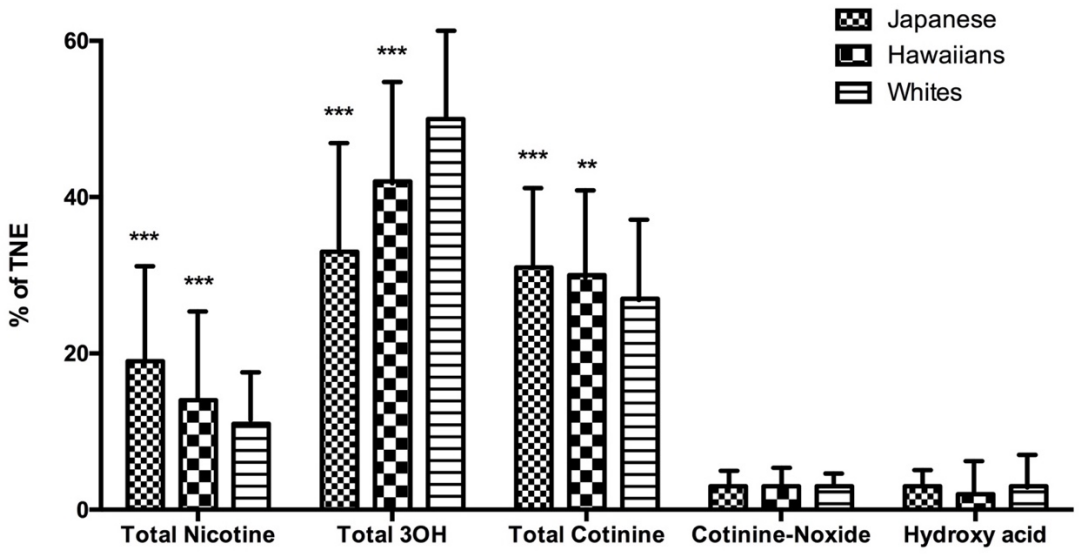

Figure 6. Proportion of total nicotine equivalents (TNE) for 5 nicotine metabolites stratified by three racial/ethnic groups.
Japanese Americans (n=81); White non-Hispanic (n=107); Native Hawaiian (n=104). (**p < 0.01, and ***p < 0.001)

3.2.2 The difference in metabolites among the people with different P450 2A6 activity

3.2.2.1 The difference in distribution of metabolites that make up TNE among the people with different P450 2A6 activity

The highly genetically variable enzyme P450 2A6 metabolizes nicotine to cotinine and cotinine to *trans* 3′-hydroxycotinine. The ratio of these two nicotine metabolite concentrations (3′-hydroxycotinine /cotinine), known as the nicotine metabolite ratio (NMR), is commonly used as a biomarker of P450 2A6 enzymatic activity [33]. The lower NMR reflects the low/less efficient activity of the *CYP2A6* variant and indicates a slower rate of nicotine metabolism. We selected the data of all 81 Japanese people and arranged them in ascending order of NMR. The first quartile, median, third quartile and fourth quartile mean values of NMR were 0.057, 0.118, 0.221and 0.353 respectively. Comparing the overall nicotine metabolism of Q1(slow metabolizers) and Q4(fast metabolizers), we found the final product of nicotine 5′-oxidation (3′-hydroxycotinine, green in Fig.7) increased significantly in fast metabolizers. For the hydroxy acid value which we are interested in, different P450 2A6 enzymatic activities do not result in a significant difference in the proportion of nicotine being metabolized to hydroxy acid (dark blue, Fig.7 Q1 vs Q4).

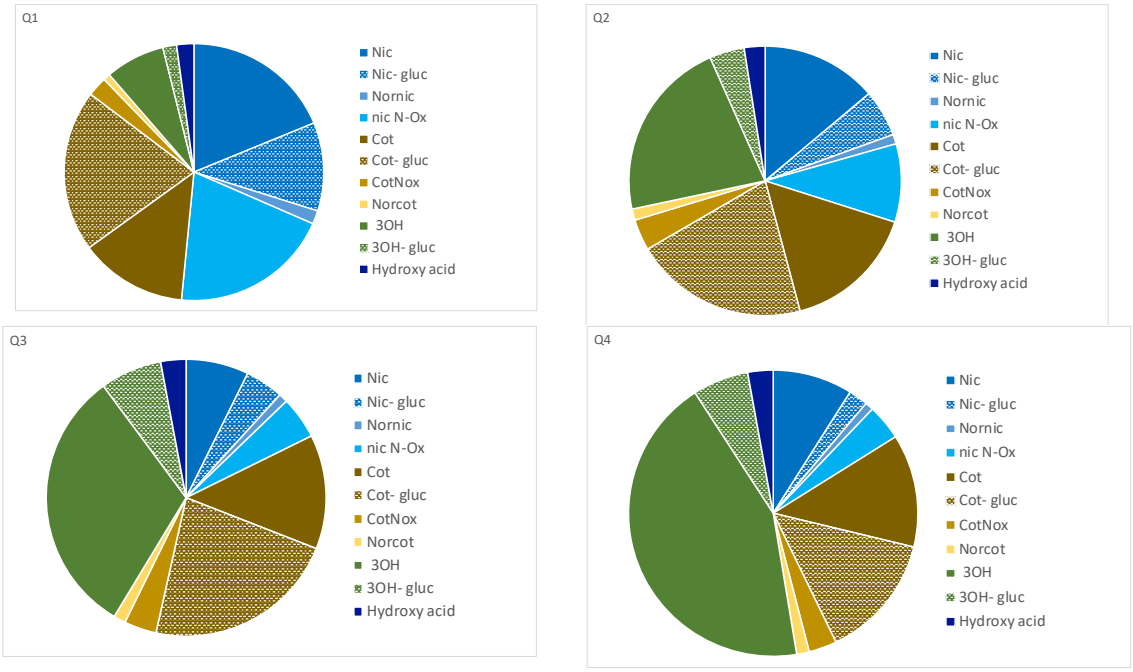

Figure 7. The distribution of urinary nicotine metabolites in four NMR quartiles.
The values for each slice of the pie are the mean percentage of the compounds excreted relative to total nicotine equivalents. The means of the NMR quartiles Q1 to Q4 were 0.057, 0.118, 0.221and 0.353.

Further, we would like to see whether smokers who have no P450 2A6 activity excrete hydroxy acid. That is, we would like to figure out if the formation of hydroxy acid depends on P450 2A6. In Fig.8, the nicotine metabolite

distribution is presented for a smoker with a *CYP2A6* whole gene deletion (sample #45) and a smoker with no P450 2A6 activity (sample #206). These smokers without P450 2A6 enzyme still excreted hydroxy acid, and its proportion was not much different compared with its proportion in smokers who have P450 2A6 activity (Fig. 7). This further confirms that there is not a significantly relationship between hydroxy acid and the P450 2A6 enzyme.

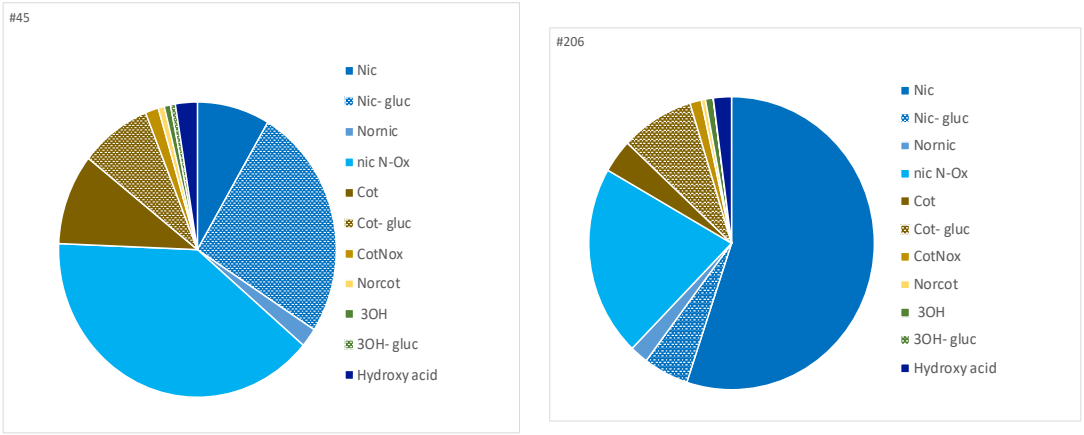

Figure 8. The distribution of urinary nicotine metabolites in two smokers without P450 2A6 activity. The values for each slice of the pie are the mean percentage of the compounds excreted relative to total nicotine equivalents. Sample #45 (CYP2A6*4/*4); sample #206 (CYP2A6*12/*12).

3.2.2.2 The difference in cotinine metabolites among the people with different P450 2A6 activity

In order to understand the role of P450 2A6 activity in cotinine metabolism, we expressed cotinine metabolism as a percentage of the sum of all cotinine metabolites (the sum of free cotinine, cotinine-*N*-glucuronide, cotinine-*N*-oxide, norcotinine and 3′-hydroxycotinine). We selected all data for 81 Japanese and arranged them in ascending order of NMR. Comparing the overall cotinine metabolism of Q1 (slow metabolizers) and Q4 (fast metabolizers), we found that the proportion of 3′-hydroxycotinine in fast metabolizers increased significantly (light blue in Fig.9). For the cotinine-*N*-oxide we are interested in, its proportion decreased significantly with more P450 2A6 activity (grey in Fig.9).

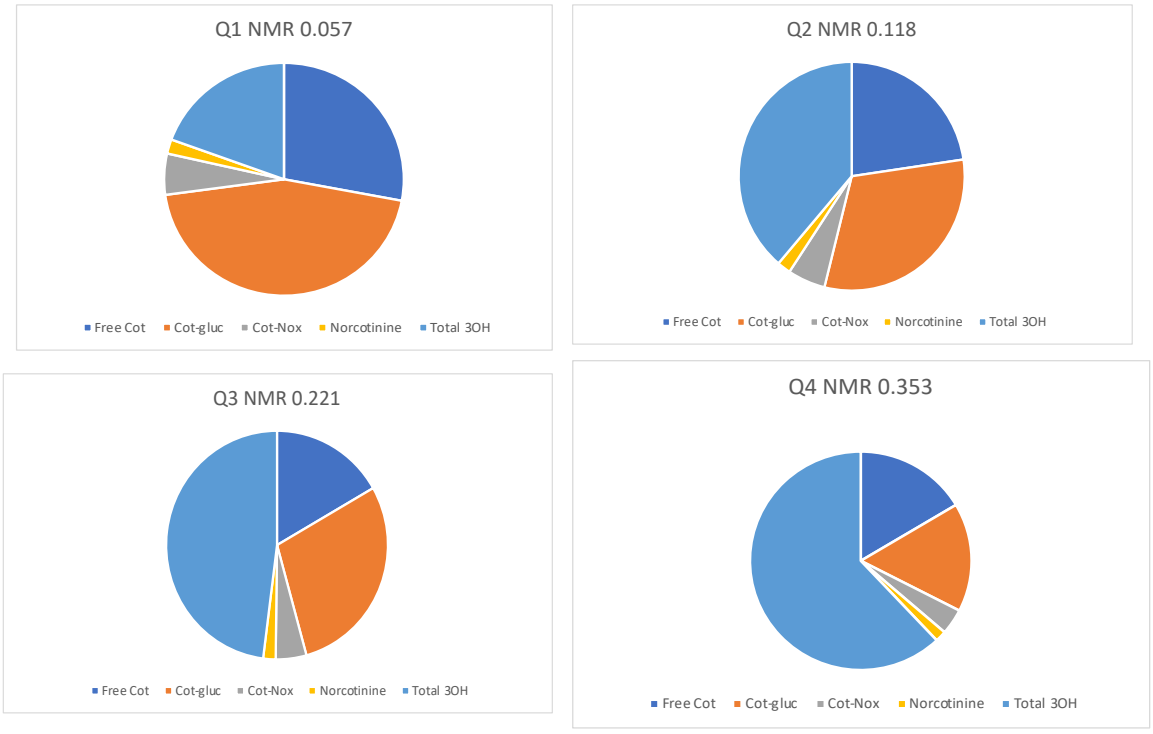

Figure 9. The distribution of urinary cotinine metabolites in four NMR quartiles. The values for each slice of the pie are the mean percentage of the compounds excreted relative to all cotinine metabolites.

From the pie charts of the distribution of cotinine metabolites for sample #45(*CYP2A6* whole gene deletion) and #206(no P450 2A6 activity), we saw that without P450 2A6 enzyme, cotinine-*N*-oxide was still excreted, and the proportion even slightly increased. It is likely that the decrease in 3′-hydroxycotinine formation with P450 2A6 activity results in more cotinine available for N-oxidation.

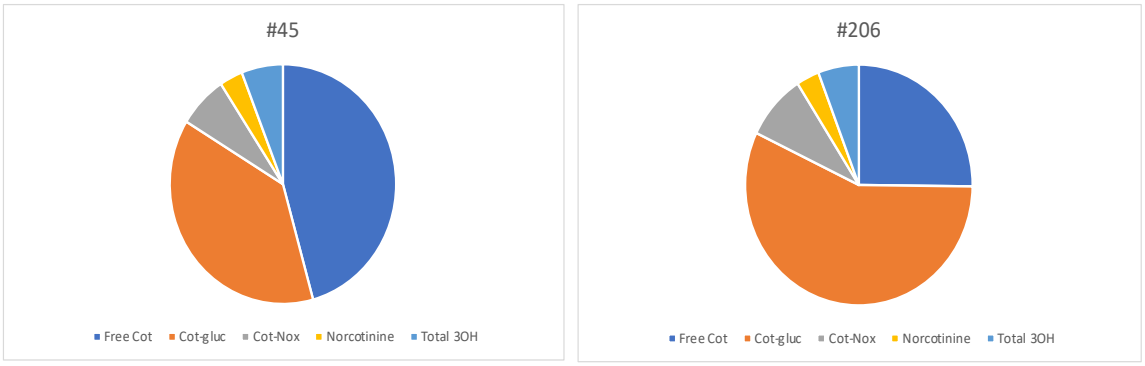

Figure 10. The distribution of urinary nicotine metabolites in two smokers without P450 2A6 activity. The values for each slice of the pie are the mean percentage of the compounds excreted relative to all cotinine metabolites. Sample #45 (CYP2A6*4/*4); sample #206 (CYP2A6*12/*12).

CHAPTER 4. CONCLUSION AND DISCUSSION

We modified an existing LC-MS/MS method for the analysis of nicotine and three metabolites to quantify four additional metabolites. The introduction of an additional solid phase extraction column allows us to measure hydroxy acid. By collecting the methanol wash and the modification of the HPLC condition for mass spectrometry, we are able to detect cotinine-*N*-oxide, nornicotine and norcotinine. Therefore, with a single sample, we can measure nicotine and 7 metabolites which can minimize potential errors that might occur if multiple samples were required. Treating the sample with or without β-glucuronidase, three additional glucuronide metabolites are measured.

In our results, hydroxy acid was around 3% of total nicotine metabolites. In 1999, the Hecht lab estimated that about 12% of nicotine was metabolized to hydroxy acid based on the relationship between the amount of hydroxy acid and cotinine measured (n=8) [16]. In that study, the hydroxy acid concentration was about 50% of the concentration of total cotinine. Surprisingly, hydroxy acid in Whites in our study was only 11% of total cotinine. Another more recent study showed that hydroxy acid accounted for 5% of total nicotine metabolites in 40 smokers[34]. In this study total cotinine accounted for 27% of total nicotine metabolites which is same percentage as

our results. Differences in results may be due to differences in methodology or the limitated number of subjects. The experiment by Hecht et al was done more than 20 years ago, and the composition of the cigarette may be slightly changed. Hydroxy acid may have other sources. Also, the Hecht study used 24 hours urine while the other two studies used morning urine or convenient spot urine. The half-life of cotinine is around 20 hours. Hydroxy acid is polar so that its half-life is likely to be much shorter.

Since P450 2A6 is key to nicotine metabolism, we selected three ethnic groups that have a range of P450 2A6 activities to study the effect of P450 2A6 activity on nicotine metabolism to hydroxy acid, and the metabolism of cotinine to cotinine-N-oxide. Previously, we already took the advantages of it to study various nicotine metabolites such as cotinine and 3′-hydroxycotinine [27]. If the molecules depend on P450 2A6 activity, we expected to see variations among the groups. In our study, we quantified the distribution of hydroxy acid and cotinine-*N*-oxide in the three ethnic groups and figured out if their formation relied on P450 2A6 activity. Experiments with human liver microsomes *in vitro* have shown that P450 2A6 can yield keto acid and hydroxy acid by 2'-hydroxylation of nicotine [15]. However, from our results described here, hydroxy acid is mostly not related to P450 2A6. And we learned that cotinine-*N*-oxide was also not likely related to P450 2A6. Other

cytochromes P450 family enzyme such as CYP2B may contribute to their formations. P450 2B metabolites nicotine and cotinine in the rat [35]. Cotinine-*N*-oxide is a metabolite of cotinine in the rat and increases when P450 2B levels are induced [36].

Analyzing all 10 metabolites, we found proportions of nicotine metabolized by 2A6 5′-oxidation, N-glucuronidation, N-oxidation and hydroxy acid in three ethnic groups. In whites, the amounts are 48%, 32%, 8% and 3%, respecitively. In Hawaiian, they are 45%, 31%, 9% and 2%. In Japanese, they are 40%, 29%, 11% and 3%. When we began these experiments, it was unknown if hydroxy acid is a P450 2A6 dependent metabolite or if it forms by an independent pathway. Since P450 2A6 does not appear to be directly related to hydroxy acid formation, we quantified this fourth pathway of nicotine metabolism independently. It is relatively minor.

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