

High-Throughput Simultaneous Analysis of Five Urinary Metabolites of Areca Nut and Tobacco Alkaloids by Isotope-Dilution Liquid Chromatography-Tandem Mass Spectrometry with On-Line Solid-Phase Extraction

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Abstract

Background: Areca nut and tobacco are commonly used drugs worldwide and have been frequently used in combination. We describe the use of on-line solid-phase extraction and isotope-dilution liquid chromatography-tandem mass spectrometry for the simultaneous measurement of five major urinary metabolites of both areca nut and tobacco alkaloids, namely, arecoline, arecaidine, *N*-methylnipecotic acid, nicotine, and cotinine.

Methods: Automated purification of urine was accomplished with a column-switching device. After the addition of deuterium-labeled internal standards, urine samples were directly analyzed within 13 minutes. This method was applied to measure urinary metabolites in 90 healthy subjects to assess areca nut/tobacco exposure. Urinary time course of arecoline, arecaidine, and *N*-methylnipecotic acid was investigated in five healthy nonchewers after oral administration of areca nut water extracts.

Results: The limits of detection were 0.016 to 0.553 ng/mL. Interday and intraday imprecision were <10%. Mean recoveries of five metabolites in urine were 97% to 114%. Mean urinary concentrations of arecoline, arecaidine, *N*-methylnipecotic acid, nicotine, and cotinine in regular areca nut chewers also smokers were 23.9, 5,816, 1,298, 2,635, and 1,406 ng/mg creatinine, respectively. Time course study revealed that after administration of areca nuts extracts, the major urinary metabolite was arecaidine with a half-life of 4.3 hours, followed by *N*-methylnipecotic acid with a half-life of 7.9 hours, and very low levels of arecoline with a half-life of 0.97 hour.

Conclusions: This on-line solid-phase extraction liquid chromatography-tandem mass spectrometry method firstly provides high-throughput direct analysis of five urinary metabolites of areca nut/tobacco alkaloids.

Impact: This method may facilitate the research into the oncogenic effects of areca nut/tobacco exposure. *Cancer Epidemiol Biomarkers Prev*; 19(10); 2570–81. ©2010 AACR.

Introduction

The areca nut (fruit of the *Areca catechu* tree) is the fourth most commonly used psychoactive substance in the world after tobacco, alcohol, and caffeine (1). It is commonly consumed by Asian populations and migrated communities living in Africa, Europe, and North America (2). Areca nut can be chewed alone or in a variety of ways

that differ by region. Betel quid contains fresh, dried, or cured areca nut, catechu (*Acacia catechu*), and slaked lime (calcium oxide and calcium hydroxide) wrapped in a betel leaf (*Piper betle*). In some countries, particularly in India, most habitual chewers of betel quid add tobacco. In Taiwan, the green unripe areca nut is often chewed with betel inflorescence, but tobacco is not added. Areca nut has also been available in commercially prepared forms in the last few decades. The product is basically a flavored and sweetened dry mixture of areca nut, catechu, and slaked lime with tobacco (gutkha) or without tobacco (pan masala; ref. 3). A previous evaluation in 1985 by the International Agency for Research on Cancer had found that chewing betel quid with tobacco (group 1) is carcinogenic to humans (4). Recently, the new evaluation goes further to conclude that chewing betel quid without tobacco (group 1) and areca nut itself (group 1) is also carcinogenic to humans (5).

There are four main areca alkaloids in areca nut: arecoline, arecaidine, guvacine, and guvacoline. Arecoline,

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (<http://cebp.aacrjournals.org>).

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doi: 10.1158/1055-9965.EPI-10-0483

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the main alkaloid present at up to 1% of dry weight, is thought to be responsible for a central cholinergic stimulation and monoamine transmission, which then activates both sympathetic and parasympathetic effects (6, 7). Arecoline has been further shown to be implicated in the pathogenesis of oral diseases because of its genotoxic, mutagenic, and carcinogenic potential (3, 8). However, relatively little is known about the metabolism of arecoline as well as other alkaloids. Until recently, Giri et al. (9) reported a metabolic map of arecoline and arecaidine in the mouse and found that the major metabolite of both arecoline and arecaidine was *N*-methylnipecotic acid. Nevertheless, except for arecoline, none of the areca nut alkaloid metabolites has ever been identified in humans.

Tobacco smoking has long been recognized as a major cause of death and disease in many countries (10). Nicotine is the major alkaloid of tobacco and is responsible for tobacco addiction. When tobacco is smoked, nicotine is efficiently absorbed into the bloodstream through the lungs and rapidly metabolized to many different compounds (e.g., cotinine, *trans*-3-hydroxycotinine, nornicotine and cotinine-*N*-oxide; ref. 11). These nicotine metabolites have been recently measured in urine to provide a better estimate of exposure to tobacco smoke. Among these metabolites, cotinine with a longer half-life of ~20 hours (12) is by far the best documented and most frequently utilized marker (13, 14).

Several chromatographic-based techniques have been developed for the measurement of tobacco alkaloids and areca nut alkaloids (mostly arecoline only) in biological samples, such as high performance liquid chromatography (HPLC) with UV detection (HPLC-UV), gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-tandem MS (LC-MS/MS; refs. 15-18). The former two methods, however, can be difficult to carry out in the clinical laboratory and are labor intensive, require time-consuming sample preparation, or exhibit inadequate specificity when used to test urine. LC-MS/MS is a relatively new and powerful technology that can overcome the sensitivity and selectivity issues in the analysis of urinary metabolites. Accurate quantification of targets at extremely low levels in matrix has frequently relied on the use of stable isotope-labeled standards to compensate for the loss of analyte during sample preparation, which has been the most critical step to eliminate the matrix effect for analysis by mass spectrometry (19). Furthermore, the on-line sample extraction using a column-switching device is an extremely useful technique to prepare biological samples automatically for LC-MS methods (20, 21). Its advantages include less ion suppression and relatively short run times, as well as higher sensitivity and selectivity, especially for urine samples containing a considerable amount of coeluting interferences.

Because of the serious health consequences of areca nut and tobacco and the fact that most areca nut users also use tobacco either in the form of chewing or smoking (3, 22), methods for a simultaneous determination of urinary metabolites of both areca nut and tobacco alkaloids

are extremely important. We report here a new, highly sensitive isotope dilution LC-MS/MS method coupled with on-line solid phase extraction (SPE) for simultaneous detection of five urinary metabolites of areca nut and tobacco alkaloids (arecoline, arecaidine, *N*-methylnipecotic acid, nicotine and cotinine). This method was applied to investigate the urinary concentrations of these five metabolites in non-areca nut chewers who are also non-smokers, non-areca nut chewers but regular cigarette smokers, and regular areca nut chewers who are also cigarette smokers. Furthermore, the urinary time course of arecoline, arecaidine, and *N*-methylnipecotic acid was firstly investigated in five healthy males after oral administration of areca nut water extracts.

Materials and Methods

Chemicals

Solvents and salts were of analytical grade. Reagents were purchased from the indicated sources: arecoline, arecaidine, nicotine, and cotinine from Sigma-Aldrich; *N*-methylnipecotic acid from Oakwood Product; the custom-made arecoline- d_3 , arecaidine- d_3 , and *N*-methylnipecotic acid- d_3 from Ryss Lab; and nicotine- d_4 and cotinine- d_3 from Cerilliant.

Preparation of water extract of areca nuts

Commercial fresh and unripe areca nuts (about the size of an olive) were purchased from a local shop in Taiwan. A total of 12 areca nuts (~36 g) were ground and suspended in 60 mL of deionized water. The mixture was stirred for 1 hour at room temperature and the extract was collected by centrifugation. This extraction procedure was repeated once more by adding 60 mL of deionized water to the residue. Both extracts were pooled and mixed well, representing 12 areca nuts (~36 g) of extracted material in 120 mL deionized water, for further use in the time course study as described in a later section.

Participants and urine samples

This study was approved by the Institutional Review Board of Chung Shan Medical University Hospital.

Cross-sectional study. Single spot urine samples were obtained from 90 apparently healthy individuals (31 non-areca nut chewers also nonsmokers, 26 non-areca nut chewers but regular cigarette smokers, and 33 regular areca nut chewers also cigarette smokers). A questionnaire was used to obtain data on subject age, body mass index (BMI), and the areca nut chewing or smoking habits (self-reported daily consumption of areca nut and/or cigarettes).

Time course study. Five healthy male volunteers who did not chew areca nut and smoke cigarettes in the past years were each administered 20 mL of water extracts of areca nut (representing two areca nuts) orally. Urine samples were collected immediately before and at different time points (2, 4, 6, 8, 10, 12, 14, 17, 24, 27, and 33 hours) after administration of the extracts. Each subject was

required to drink at least 150 mL of water after each void to assure an adequate volume of urine. Urine volume was measured at each void in a calibrated container and 10 mL fractions were saved. Urine samples from both the cross-sectional study and the time course study were kept at 4°C during sampling, and stored at -20°C prior to analysis. Urinary creatinine was also measured for each sample using a HPLC-UV method described by Yang (23).

Simultaneous analysis of five urinary metabolites of areca nut/tobacco alkaloids using on-line SPE LC-MS/MS

Preparation of urine samples. The urine samples were thawed, vortexed, and then heated to 37°C for 10 minutes to release possible alkaloid metabolites from precipitate. After centrifugation at 5,000 g, 20 µL of urine were diluted 10 times with a solution containing 2 ng each of arecoline-d₃, arecaidine-d₃, N-methylnipecotic acid-d₃, and cotinine-d₃, and 4 ng of nicotine-d₄ as internal standards in 2% (v/v) methanol containing 0.1% (v/v) trifluoroacetic acid (TFA).

A primary standard stock solution mixture of five analytes (1,000 µg/mL) was prepared by dissolving the same amount of each analyte in 10% (v/v) methanol and then further diluting with 2% (v/v) methanol/0.1% (v/v) TFA to yield appropriate working solutions. Calibrators were made in drug-free pooled urine and prepared by spiking with a known amount of standard mixture, followed by 1:1 serial dilution with drug-free urine to obtain upper

and lower calibrators. Calibrators were then processed and analyzed as urine samples. Two linear ranges were determined for arecoline, arecaidine, N-methylnipecotic acid, and cotinine, from 0.006 to 0.375 ng (low range: 0.006, 0.012, 0.023, 0.047, 0.094, 0.188, and 0.375 ng) and from 0.375 to 24 ng (high range: 0.375, 0.75, 1.5, 3.0, 6.0, 12, 24 ng), whereas the ranges from 0.094 to 1.5 ng and from 1.5 to 24 ng were applied for nicotine.

Automated on-line SPE. The column-switching system used in this study was as described in detail elsewhere (21). It consisted of a switching valve (two-position microelectric actuator from Valco) and a C18 trap column (75 × 2.1 mm i.d., 5 µm, ODS-3, Inertsil). The switching valve function was controlled by PE-SCIEX control software (Analyst, Applied Biosystems). The column-switching operation, including the LC gradients used during the on-line cleanup and the analytical procedures, is summarized in detail in Table 1. When the switching valve was at position A, 50 µL of prepared urine sample were loaded on the trap column by an autosampler (Agilent 1100 series, Agilent Technology), and a binary pump (Agilent 1100 series, Agilent Technology) delivered the 2% (v/v) methanol/0.1% (v/v) TFA at a flow rate of 200 µL/minute as the loading and washing buffer (solvent Ia). After the column was flushed with the loading buffer for 1.8 minutes, the valve switched to the injection position (position B) to inject the sample into the LC system. At 5 minutes after injection (Table 1), the valve was switched back to position A, and the trap column was washed with a mobile phase (eluent I) with a gradient

Table 1. Timetable for the column-switching procedure

Time	Eluent I (trap column)		Eluent II (analytical column)		Valve position	Flow rate, µL/min	Remarks
	Solvent Ia,* %	Solvent Ib,† %	Solvent IIa,‡ %	Solvent IIb,§ %			
0	100	0	100	0	A	200	Injection and washing of sample
1.8	100	0	100	0	B	200	Start of elution to the analytical column
5.0	100	0	100	0	A	200	End of elution; trap column cleanup and reconditioning
5.1	0	100	100	0	A	200	
7.0	0	100	83	17	A	200	
7.1	100	0	83	17	A	200	
9.0	100	0	83	17	A	200	
9.1	100	0	0	100	A	200	
11.0	100	0	0	100	A	200	
11.1	100	0	100	0	A	200	
13.0	100	0	100	0	A	200	

*2% (v/v) methanol containing 0.1% (v/v) TFA.

†75% (v/v) methanol containing 0.1% (v/v) TFA.

‡2% (v/v) methanol containing 0.1% (v/v) FA.

§50% (v/v) methanol containing 0.1% (v/v) FA.

Compound	Structure	Q1 mass, amu	Q3 mass, amu	Dwell time, ms	DP, † V	CE, ‡ V	FP, § V
Arecoline		156	44	50	30	35	150
		156	113	50	30	30	150
Arecoline-d ₃		159	47	50	40	30	200
Arecaidine		142	44	50	40	25	200
		142	99	50	40	20	200
Arecaidine-d ₃		145	47	50	40	30	200
N-methylnipecotic acid		144	98	50	40	25	200
		144	126	50	40	25	200
N-methylnipecotic acid-d ₃		147	73	50	42	25	200
Nicotine		163	132	50	30	25	150
		163	106	50	30	25	150
		167	136	50	40	25	180
Cotinine		177	80	50	30	35	200
		177	98	50	30	35	200
		180	80	50	40	33	160

* Deuterium-isotope labeled position
† Declustering potential
‡ Collision energy
§ Focusing potential
|| Quantifier transition

Figure 1. Chemical structure and tandem mass spectrometry parameters for five urinary metabolites of areca nut/tobacco alkaloids.

from 100% solvent Ia to 100% solvent Ib (75% methanol/0.1% TFA; see Table 1), followed by 100% solvent Ia for 5.9 minutes for equilibration of the column and preparation for the next analysis. The total run time was 13 minutes.

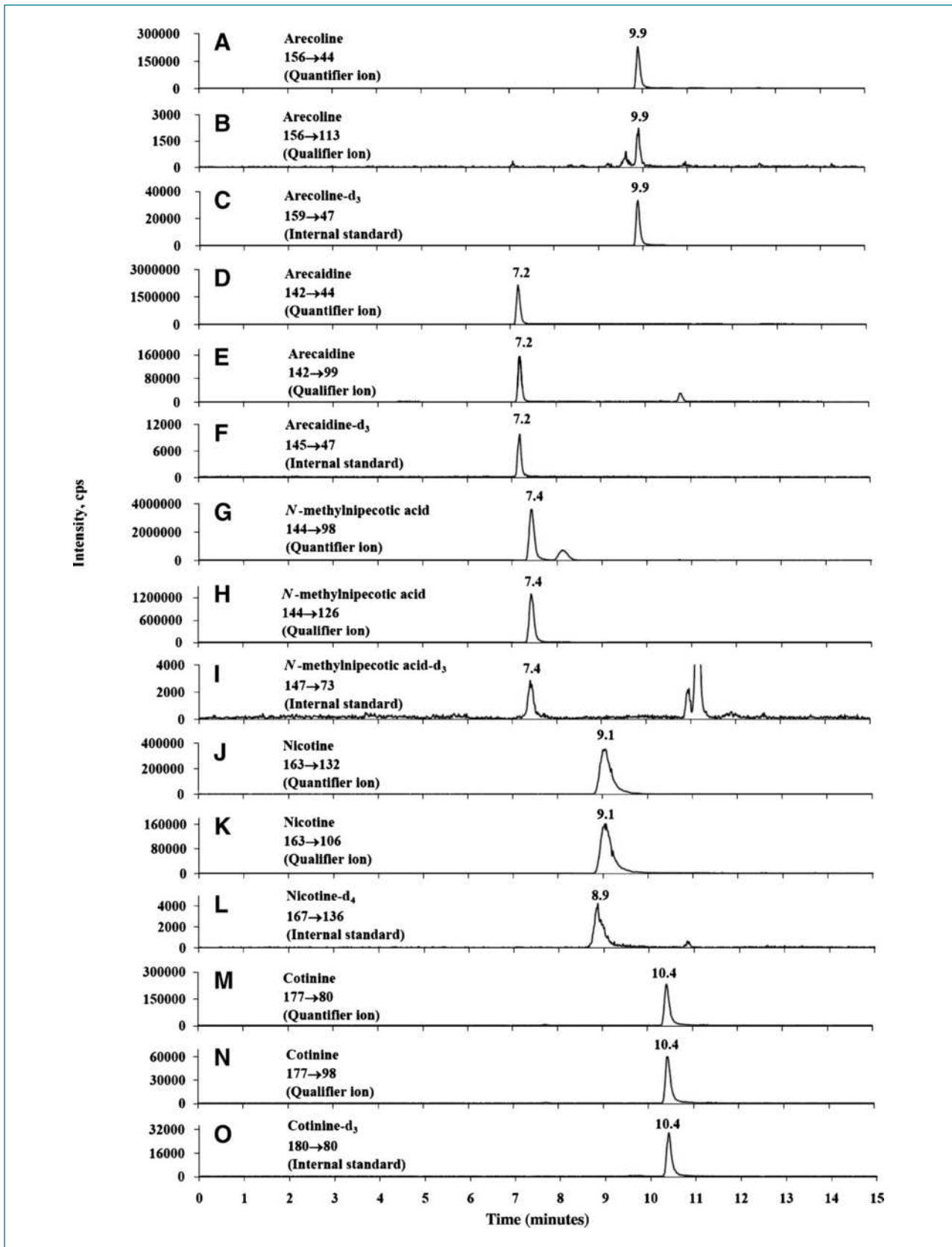
Liquid chromatography. After automatic sample clean-up (see Table 1 at the 1.8-minute time point), the sample was automatically transferred onto a C18 column (250 × 2.1 mm i.d., 5 μm, ODS-3, Inertsil). The mobile phase was 2% (v/v) methanol containing 0.1% (v/v) formic acid (FA; solvent IIa) and was delivered at a flow rate of 200 μL/minute. At 7.0 minutes after injection, the mobile phase was varied to 83% solvent IIa for 2 minutes, followed by 100% solvent IIb (50% methanol/0.1% FA) for 1.9 minutes and rapidly back to 100% solvent IIa.

Electrospray ionization MS/MS. The sample eluting from the HPLC system was introduced into a TurboIon-spray source installed on an API 3000 triple-quadrupole mass spectrometer (Applied Biosystems), operated in positive mode with a needle voltage of 5.5 kV, nitrogen as the nebulizing gas, and turbogas temperature set at 450°C. Data acquisition and quantitative processing were

accomplished with Analyst software, ver. 1.4 (Applied Biosystems). For all analytes, the [M+H]⁺ ion was selected by the first mass filter. After collisional activation, two fragment ions were selected: the most abundant fragment ion was used for quantification (quantifier ion), and the second most abundant ion was used for qualification (qualifier ion). For the stable isotope-labeled internal standards only one fragment ion was selected. The chemical structure and optimization results for each analyte in multiple reaction monitoring scan mode are given in Fig. 1. Source parameters were as follows: nebulizer gas flow, 10; curtain gas flow, 10; collision-assisted-dissociation gas flow, 12; turbo gas flow, 8. Peak full-width at half-maximum was set to 0.7 Th (Thomson = 1 amu per unit charge) for both Q1 and Q3.

Statistical methods

Mean and SD were used to describe the distributions of urinary metabolites and the demographic data for study subjects. The data were analyzed using the SAS statistical package (SAS, version 9.1). Mann-Whitney *U* test was used to compare the continuous variables



among groups. Spearman correlation coefficients were used to study the relationship of urinary metabolites concentrations to self-reported daily areca nut (or cigarette) consumption. Multiple linear regression models were used to investigate the relationship of urinary metabolites concentrations to self-reported daily areca nut (or cigarette) consumption after adjusting for other variables (i.e., age and BMI).

Results

On-line SPE LC-MS/MS analysis of five urinary metabolites of areca nut/tobacco alkaloids in human urine

Chromatography and mass spectra. A typical on-line SPE LC-MS/MS chromatogram for five urinary metabolites and their isotope internal standards of an areca nut chewer also cigarette smoker is shown in Fig. 2. The retention time of arecoline was 9.9 minutes. The positive electrospray ionization (ESI) mass spectrum of arecoline contained a $[M+H]^+$ precursor ion at m/z 156 and product ions at m/z 44 (quantifier ion, Fig. 2A) and m/z 113 (qualifier ion, Fig. 2B) due to loss of $C_6H_8O_2$ or C_2H_5N ; a precursor ion at m/z 159 and product ion at m/z 47 characterized the arecoline- d_3 (Fig. 2C). For arecaidine, the retention time was 7.2 minutes. The $[M+H]^+$ precursor ion of arecaidine was at m/z 142 and product ions appeared at m/z 44 (quantifier ion, Fig. 2D) and m/z 99 (qualifier ion, Fig. 2E), resulting from the loss of $C_5H_6O_2$ or C_2H_5N ; a precursor ion at m/z 145 and product ion at m/z 47 characterized the arecaidine- d_3 (Fig. 2F). Meanwhile, the retention time for *N*-methylnipecotic acid was 7.4 minutes. Its $[M+H]^+$ precursor ion was at m/z 144 and product ions appeared at m/z 98 (quantifier ion, Fig. 2G) and m/z 126 (qualifier ion, Fig. 2H) formed by the loss of CH_2O_2 or H_2O ; a precursor ion at m/z 147 and product ion at m/z 73 characterized the *N*-methylnipecotic acid- d_3 (Fig. 2I). Because serious coeluting interference was observed at the first and second most abundant fragment ions (m/z 101 and 129), a third abundant fragment ion (m/z 73) was used for *N*-methylnipecotic acid- d_3 . In terms of nicotine, the retention time was 9.1 minutes. However, a slight difference in retention time between the analyte (9.1 minutes) and its deuterated internal standard (8.9 minutes) was observed. The retention time difference could be attributed to the altered hydrophilic nature of the nicotine internal standard labeled with four deuterium atoms, which was known as "deuterium isotope effect" during reversed phase LC separation (24). The $[M+H]^+$ precursor ion of nicotine was at m/z 163 and gave product ions at m/z 132 (quantifier ion, Fig. 2J) and m/z 106 (qualifier

ion, Fig. 2K) corresponding to loss of the CH_5N or C_3H_7N ; a precursor ion at m/z 167 and product ion at m/z 136 characterized the nicotine- d_4 (Fig. 2L). For cotinine, the retention time was 10.4 minutes. The $[M+H]^+$ precursor ion of cotinine was at m/z 177 and its product ions were at m/z 80 (quantifier ion, Fig. 2M) and m/z 98 (qualifier ion, Fig. 2N) corresponding to loss of the C_5H_7NO or C_5H_5N ; a precursor ion at m/z 180 and product ion at m/z 80 characterized the cotinine- d_3 (Fig. 2O). The transitions obtained for the ESI/MS-MS analysis of nicotine and cotinine were in agreement with those previously reported (17, 25, 26).

Limit of quantification and limit of detection. The limit of quantification was defined as the lowest concentration in urine that could be reliably and reproducibly measured with values for accuracy, intraday imprecision, and interday imprecision <20%. Using the present method, the limits of quantification were determined to be 0.05, 0.30, 0.09, 1.80, and 0.17 ng/mL on column (2.5, 15, 4.5, 90 and 8.5 pg in an injection volume of 50 μ L) for arecoline, arecaidine, *N*-methylnipecotic acid, nicotine, and cotinine, respectively, based on direct measurement of diluted calibration solutions. The limits of detection (LOD) in urine, defined as the lowest concentration that gave a signal-to-noise ratio of at least 3, were 0.016, 0.078, 0.037, 0.553, and 0.028 ng/mL on column (0.8, 3.9, 1.85, 27.7, and 1.4 pg) for arecoline, arecaidine, *N*-methylnipecotic acid, nicotine, and cotinine, respectively.

Linearity, precision, accuracy, and recovery. Two linear calibration curves covering the low concentration range (0.006–0.375 ng for arecoline, arecaidine, *N*-methylnipecotic acid, and cotinine, and 0.094–1.5 ng for nicotine) and the high concentration range (0.375–24 ng for arecoline, arecaidine, *N*-methylnipecotic acid, and cotinine, and 1.5–24 ng for nicotine) were obtained by serial dilution of calibrators with drug-free urine. Each calibrator contained 2 ng each of arecoline- d_3 , arecaidine- d_3 , *N*-methylnipecotic acid- d_3 , and cotinine- d_3 and 4 ng of nicotine- d_4 . Linear regression was calculated with nonweighting and non-zero-forced, and the linear equations of each analyte are summarized in Table 2. The correlation coefficients (r^2) obtained were >0.99 in all cases. Over the entire concentration range of the calibration curves, the mean observed percentage deviation of back-calculated concentrations was between –15.2% and +9.6% with an imprecision (CV) <15%. For each metabolite in urine, the peak identity was also confirmed by comparing the peak area ratios (quantifier/qualifier) with those of the calibrators. As an acceptance criterion, ratios in urine samples should not deviate by more than \pm 25% from the mean ratios in the calibrators.

Figure 2. Chromatograms of five urinary metabolites of areca nut/tobacco alkaloids of a regular areca nut chewer who is also a smoker using LC-MS/MS coupled with on-line SPE. Multiple reaction monitoring transitions of m/z 156→44 (A) and m/z 156→113 (B) for arecoline and m/z 159→47 (C) for arecoline- d_3 ; m/z 142→44 (D) and m/z 142→99 (E) for arecaidine and m/z 145→47 (F) for arecaidine- d_3 ; m/z 144→98 (G) and m/z 144→126 (H) for *N*-methylnipecotic acid and m/z 147→73 (I) for *N*-methylnipecotic acid- d_3 ; m/z 163→132 (J) and m/z 163→106 (K) for nicotine and m/z 167→136 (L) for nicotine- d_4 ; m/z 177→80 (M) and m/z 177→98 (N) for cotinine and m/z 180→80 (O) for cotinine- d_3 .

Table 2. Linearity, precision and recovery

Compound	Linearity Linear equation	r^2	Precision* mean (SD), ng/mL		
			Intraday		
			Urine 1	Urine 2	Urine 3
Arecoline	L: $y = 4.2497x + 0.0063$	0.9996	26.8 (1.1)	102.0 (1.4)	512.4 (8.5)
	H: $y = 3.9874x + 0.8203$	0.9992	4.1 [†]	1.4	1.7
Arecaidine	L: $y = 0.8285x + 0.0037$	0.9991	26.1 (1.6)	109.2 (2.1)	491.0 (5.7)
	H: $y = 1.0589x - 0.4762$	0.9999	6.1	1.9	1.2
<i>N</i> -methylnipecotic acid	L: $y = 25.869x + 0.1409$	0.9987	23.7 (1.7)	100.1 (4.6)	481.2 (15.3)
	H: $y = 25.763x + 4.6149$	0.9994	7.2	4.6	3.2
Nicotine	L: $y = 1.8379x + 0.0664$	0.9994	27.0 (2.3)	106.0 (3.0)	500.0 (6.7)
	H: $y = 1.3872x + 1.3527$	0.9980	8.5	2.8	1.3
Cotinine	L: $y = 0.9702x + 0.0062$	0.9992	26.7 (1.2)	104.5 (2.9)	492.0 (13.8)
	H: $y = 0.8523x + 0.4059$	0.9990	4.5	2.8	2.8

(Continued on the following page)

The precision and accuracy of the present method were evaluated by spiking drug-free urine sample with unlabeled standard mixture at three different concentrations (25 ng/mL for urine 1, 100 ng/mL for urine 2, and 500 ng/mL for urine 3; each standard mixture contained equal amounts of each analyte) and repeatedly measuring the five analytes in these three urine samples. The intraday and interday CVs were 1.2% to 8.5% and 1.0% to 8.1%, respectively. Intraday and interday accuracy were 95% to 109% and 94% to 110%, respectively.

Recovery was evaluated by adding unlabeled standard mixture at five concentrations (6.25, 12.5, 50, 100, 200 ng/mL) to a urine sample that initially contained 25 ng/mL of each analyte (urine 1), and measuring three replicates of these samples. As shown in Table 2, the mean recoveries were 97% to 114% as estimated from the increase in the measured concentration after addition of the analyte divided by the concentration that was added, whereas the recoveries as calculated from the slope of the regression were 95% to 104% ($r^2 > 0.99$).

Matrix effects. Matrix effects were calculated from the peak areas of the internal standard added to the standard mixture solutions (prepared in 2% methanol containing 0.1% TFA) and compared with the peak areas of the internal standard that was added to each urinary sample. The relative change in peak area of the internal standard was attributed to matrix effects, which reflect both on-line extraction losses and ion suppression due to the urinary matrix. In this study, the matrix effects for five metabolites were <30% in all urine samples. Although the use of stable isotope-labeled internal standards could have compensated for different matrix effects, a low matrix effect achieved in this study ensures a high sensitivity of the method (27).

Urinary excretion of five metabolites of areca nut/tobacco alkaloids

A total of 90 healthy subjects were recruited into the cross-sectional study, including 31 non-areca nut chewers also nonsmokers, 26 non-areca nut chewers but regular cigarette smokers, and 33 regular areca nut chewers also cigarette smokers. The characteristics of the participants and the five urinary metabolites concentrations are summarized in Table 3. The three groups were similar in age and BMI ($P > 0.05$, by Mann-Whitney *U* test). As for the urinary metabolites adjusted for urinary creatinine, the group of non-areca nut chewers also nonsmokers had mean urinary nicotine and cotinine concentrations of 0.9 and 3.1 ng/mg creatinine, respectively, and nondetectable concentrations of areca nut alkaloids. The group of smokers but nonchewers had a self-reported mean consumption of 17.6 cigarettes/day and mean urinary nicotine and cotinine of 1,514 and 820 ng/mg creatinine, respectively, and nondetectable concentrations of areca nut alkaloids. For the group of areca nut chewers also smokers, they reported mean daily consumptions of 23.0 cigarettes and 28.0 areca nuts and had mean urinary concentrations of arecoline, arecaidine, *N*-methylnipecotic acid, nicotine, and cotinine of 23.9, 5,816, 1,298, 2,635, and 1,406 ng/mg creatinine, respectively. The association between self-reported daily areca nut (or cigarette) consumption and the corresponding urinary metabolites was further analyzed using Spearman correlation coefficients. The urinary concentrations of arecoline, arecaidine, and *N*-methylnipecotic acid as well as the sum of these three metabolites were found to be associated with the self-reported number of areca nut chewed per day ($n = 33$; $r = 0.71$, $P < 0.01$ for arecoline; $r = 0.59$, $P < 0.01$ for arecaidine; $r = 0.56$, $P < 0.01$ for *N*-methylnipecotic acid; and $r = 0.60$, $P < 0.01$ for the sum of three metabolites; see Supplementary Fig. S1). In multiple linear regressions, these correlations between

Table 2. Linearity, precision and recovery (Cont'd)

Precision* mean (SD), ng/mL			Recovery† mean (SD), %
Interday			
Urine 1	Urine 2	Urine 3	Urine 1
25.9 (1.0)	100.9 (2.3)	500.7 (38.5)	107 (9.3)
3.9	2.3	7.7	0.95 [§]
25.7 (1.4)	109.8 (3.4)	494.5 (12.7)	107 (11.1)
5.4	3.1	2.6	1.03
23.6 (1.9)	96.0 (5.4)	482.0 (19.8)	97.2 (18.2)
8.1	5.6	4.1	0.95
24.2 (1.3)	99.0 (3.0)	497.2 (5.0)	114 (5.3)
5.4	3.0	1.0	0.98
24.7 (0.8)	98.9 (2.0)	501.9 (14.1)	106 (8.4)
3.2	2.0	2.8	1.04

Abbreviations: L, low; H, high.

*Drug-free pooled urine samples were individually spiked with unlabeled standard mixture (containing equal amounts of each analyte) at three different concentrations (25 ng/mL for urine 1, 100 ng/mL for urine 2, and 500 ng/mL for urine 3). Each urine analysis was repeated five times for the intraday and interday tests; the interday test was carried out over a period of 50 days.

†Recovery of the analytes in urine was estimated by the addition of unlabeled standard mixture at five different concentrations (6.25, 12.5, 50, 100, 200 ng/mL) to a urine sample (urine 1). The recovery was estimated from the increase in measured concentration after addition of the analyte divided by the concentration that was added.

‡CV, %.

§Recovery was estimated from the slope of the regression of the measured concentration versus the added concentration.

urinary metabolites of areca nut alkaloids and daily consumption of areca nut were not confounded by other variables, including age and BMI ($P < 0.01$). For the urinary metabolites of tobacco alkaloids, there was no correlation between urinary nicotine or cotinine (or the sum of nicotine and cotinine) and the self-reported daily cigarette consumption for the smokers ($n = 59$; $r = 0.05$, $P = 0.73$ for nicotine; $r = 0.24$, $P = 0.07$ for cotinine; and $r = 0.08$, $P = 0.53$ for the sum of the two metabolites). Multiple linear regression analysis also revealed no significant correlation between urinary nicotine metabolites and daily cigarette consumption after adjustment for age and BMI ($P = 0.67$).

Time course of three metabolites of areca nut alkaloids in human urine

Five male volunteers were each orally administered 20 mL of water extract of areca nuts, and urine samples were collected at 0 (predose), 2, 4, 6, 8, 10, 12, 14, 17, 24, 27, and 33 hours after dosing. The results of LC-MS/MS analysis revealed that 20 mL of water extract of areca nuts (representing two areca nuts) contained 1,838 μg (11.9 μmole) of arecoline, 968 μg (6.87 μmole) of arecaidine, and 82 μg (0.57 μmole) of *N*-methylnipecotic acid. Surprisingly, areca nut itself also contained *N*-methylnipecotic acid, which has not been reported previously. Figure 3 shows rapid formations of areca nut alkaloids in urine after the administration of water extract of areca nuts. The levels of arecoline (Fig. 3A) and

arecaidine (Fig. 3B) were dramatically increased from 0 to the highest concentrations of 1.84 and 1,306 ng/mg creatinine, respectively, within 2 to 4 hours and decreased gradually. By 12 hours after administration, the levels of arecoline were nondetectable in all urine samples whereas the mean level of arecaidine decreased by almost 93% (89.5 ng/mg creatinine at 12 hours) and remained decreased throughout the experiment (8.49 ng/mg creatinine at 33 hours). In terms of *N*-methylnipecotic acid (Fig. 3C), the mean level was significantly increased from 0 to the highest concentration of 304.5 ng/mg creatinine within 6 hours. The mean level of *N*-methylnipecotic acid was then decreased by 41% (179.2 ng/mg creatinine) at 8 hours and slightly increased again at 10 hours and decreased gradually thereafter. By 33 hours, the mean level of *N*-methylnipecotic acid was decreased by 92% (23.6 ng/mg creatinine). Furthermore, by the end of the experiment, it was found that the major urinary metabolite was arecaidine with a total excretion of 4.3 to 6.2 μmole , followed by *N*-methylnipecotic acid of 1.3–2.9 μmole and arecoline of 0.004–0.008 μmole for five male volunteers.

For the urinary half-life of each metabolite, semilogarithmic mean urinary excretion concentrations versus time curves were constructed for each metabolite excretion (data not shown). The urinary elimination rate constant of each metabolite was calculated by linear regression of the linear portions of these curves (28). Correlation coefficients in the regression analysis for

Table 3. Overall characteristics of the study participants

Variables	Non-areca nut chewers also nonsmokers	Non-areca nut chewers but cigarette smokers	Areca nut chewers also cigarette smokers
No.	31	26	33
Age, years			
Mean (SD)	43.0 (13.2)	43.6 (11.1)	37.9 (9.6)
Range	21-63	25-62	24-59
BMI, kg/m ²			
Mean (SD)	25.1 (3.4)	23.4 (3.2)	24.5 (4.1)
Range	17.4-31.6	18.6-29.4	17.4-29.3
Cigarettes/day			
Mean (SD)	0	17.6 (9.3)	23.0 (11.0)
Range		5-40	5-60
Areca nuts/day			
Mean (SD)	0	0	28.0 (23.8)
Range			3-100
Arecoline, ng/mg creatinine			
Mean (SD)	ND	ND	23.9 (39.3)
Range			ND-141.8*
Arecaidine, ng/mg creatinine			
Mean (SD)	ND	ND	5,816 (12,541)
Range			7.2-66,053
<i>N</i> -methylnipecotic acid ng/mg creatinine			
Mean (SD)	ND	ND	1,298 (2,580)
Range			0.8-13,833
Nicotine, ng/mg creatinine			
Mean (SD)	0.9 (1.8)	1,514 (1,330)	2,635 (3,078)
Range	ND-7.8 [†]	173-5,514	0.6-12,563
Cotinine, ng/mg creatinine			
Mean (SD)	3.1 (3.0)	820 (460)	1,406 (1,496)
Range	ND-10 [†]	314-2,406	41.9-6,423

Abbreviation: ND, not detectable.

*One out of 33 (3%) urine samples had a nondetectable level of arecoline.

[†]Out of 31 urine samples, 23 (74%) and 7 (23%) urine samples had nondetectable levels of nicotine and cotinine, respectively.

these metabolites ranged from 0.96 to 0.99, indicating a good fit of the first-order reactions. The corresponding urinary half-life of elimination ($t_{1/2}$ of elimination) was calculated from the elimination rate constant (k) according to the equation: $t_{1/2}$ of elimination = 0.693/ k . The half-lives of arecoline, arecaidine, and *N*-methylnipecotic acid were found to be 0.97, 4.3, and 7.9 hours, respectively.

Discussion

We have developed a rapid, specific, and sensitive isotope-dilution LC-MS/MS method incorporating on-line SPE and isotopic internal standards that can simultaneously detect five urinary metabolites of areca nut/tobacco alkaloids with the LODs of 0.016 to 0.553 ng/mL on column (0.8-27.7 pg) and a total analysis time per sample as short as 13 minutes.

LC-MS/MS has received a great deal of attention these years because it can provide a sensitive and selective means for comprehensive measurement of multiple metabolites. Tuomi et al. (29) and Xu et al. (30) described LC-MS/MS methods involving an off-line (manual) SPE cleanup for nicotine and cotinine and had detection limits of 1 to 10 ng/mL and 0.1 to 1 ng/mL, respectively. Similarly, the method described by Heavner et al. (31) involved also an off-line SPE and had detection limits of 4.4 and 3.7 ng/mL for nicotine and cotinine, respectively. In terms of the metabolite measurement of areca nut alkaloids, previous methods were mostly focused on the quantification of arecoline alone. Pichini et al. (18) and Zhu et al. (32) developed the ion trap LC-MS methods following a two-step liquid-liquid extraction purification or off-line SPE purification, and reported LODs of 0.4 and 8 ng/mL for arecoline, respectively. Apparently, the method established in the present

work, which involves on-line sample cleanup/purification coupled to isotope dilution LC-MS/MS, has lower LODs (0.553 ng/mL for nicotine, 0.028 ng/mL for cotinine, and 0.016 ng/mL for arecoline) than these previously reported methods. More importantly, our

method provides direct and simultaneous determination of major urinary metabolites of both areca nut and tobacco alkaloids, which would be used as a high-throughput tool to monitor the subjects with either one or both habits in research and clinical practice.

Although there are as many as 600 million chewers of areca nut products worldwide, relatively little is known about the metabolism of areca nut alkaloids. To the best of our knowledge, in the past 40 years, only several rodent studies (9, 33, 34) and one clinical trial with arecoline in Alzheimer patients (16) have been reported. This compares very poorly with nicotine, for which more than 1,000 papers have been published and 24 metabolites have been identified (11). One of the most significant metabolism studies of areca nut alkaloids in rodent is that of Giri et al. (9). With the use of a metabolomic approach, 11 metabolites of arecoline were identified in mice urine and 6 of these metabolites were shared with arecaidine. Among these metabolites, *N*-methylnipecotic acid was found to be the major metabolite of both arecoline and arecaidine after arecoline (or arecaidine) administration. Interestingly, in this study, *N*-methylnipecotic acid and arecaidine were for the first time identified in the human urine of areca nut chewers in addition to arecoline (Table 3 and Fig. 3).

In the cross-sectional study (Table 3), all of the urine samples from areca nut chewers presented quantifiable amounts of *N*-methylnipecotic acid (range, 1.9-21,534 ng/mL) as well as arecaidine (4.6-102,800 ng/mL) with the concentrations well above the LODs of our method. It is worth noting that the urine samples contained relatively low to nondetectable levels of arecoline for areca nut chewers although arecoline is the major alkaloid in areca nuts (6). This may be due to the fact that arecoline has a relatively short half-life in urine and could be barely detectable after 12 hours of oral administration, as shown in Fig. 3. Moreover, there were significant correlations between self-reported daily areca nut consumption and the corresponding urinary metabolites (i.e., arecoline, arecaidine, and *N*-methylnipecotic acid or the sum of three metabolites), suggesting that these three metabolites are quantitatively representative biomarkers for assessing the exposure to areca nut alkaloids. In terms of the urinary nicotine/cotinine measurement (Table 3), the mean values of the nicotine and cotinine concentrations for the groups of nonsmokers and smokers are similar with previously reported ranges (e.g., nicotine of >30 ng/mg creatinine and cotinine of >100 ng/mg creatinine for smokers; refs. 13, 35). However, unlike urinary metabolites of areca nut alkaloids, there was no significant correlation between self-reported daily cigarette consumption and urinary nicotine or cotinine as well as the sum of these two metabolites for smokers. Despite the possibility of recall bias in questionnaire studies, the lack of such a correlation might be because nicotine found in cigarette smoke varies substantially from brand to brand (e.g., 0.1-2.3 mg/cigarette; ref. 36); however, it may not be the case for areca nuts because the alkaloids contents

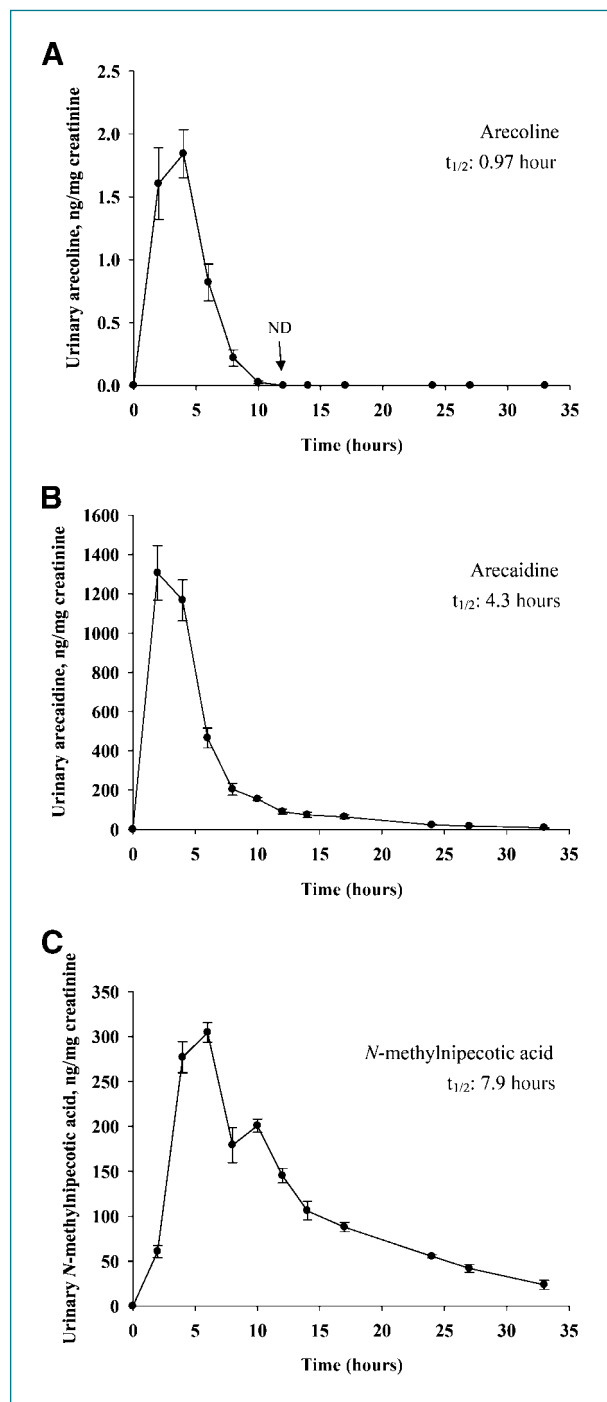


Figure 3. Time course of three urinary metabolites of areca nut alkaloids after administration of water extract of areca nuts. A, arecoline. B, arecaidine. C, *N*-methylnipecotic acid. Points, mean; bars, SE.

were quite similar in the commercial-size unripe areca nuts (e.g., ~7 mg/g of arecoline; ref. 37). Moreover, a wide variability in smoking behavior (e.g., puffing volume and duration and/or inhalation depth) may have affected the nicotine intake per cigarette smoked (38, 39) and this most likely could have interfered with the correlation analysis between self-reported cigarette consumption and urinary nicotine or cotinine.

Previous studies have shown that arecoline readily crosses the blood-brain barrier and metabolizes rapidly ($t_{1/2}$ 0.95 minute in plasma) after its i.v. administration to subjects with Alzheimer's disease (16). In this study, the results of time course measurement also revealed a rapid metabolism of this compound after oral administration of areca nut water extracts with an estimated half-life of 0.97 hour in urine. Meanwhile, relatively longer half-lives were obtained for arecaidine ($t_{1/2}$ 4.3 hours) and *N*-methylnipecotic acid ($t_{1/2}$ 7.9 hours). The increasing half-lives of arecoline < arecaidine < *N*-methylnipecotic acid estimated in this study could further support the existence of a metabolic pathway identified in a mouse model by Giri et al. (9), in which arecoline is firstly hydrolyzed to arecaidine and then undergoes carbon-carbon double-bond reduction to yield *N*-methylnipecotic acid.

Furthermore, the results of the time course study also showed that arecaidine was the major metabolite in human urine after administration of areca nut water extracts, composing 23% to 33%, of the dose administered (as calculated by total excreted arecaidine divided by total amount of arecoline and arecaidine in the water extract of areca nuts), followed by *N*-methylnipecotic acid at 6.7% to 15% (total excreted *N*-methylnipecotic acid divided by total amount of three areca nut alkaloids in the water extract) and arecoline at 0.03% to 0.07% (total excreted arecoline divided by total arecoline in the water extract). Our finding was slightly different from the previous study in mouse (9), showing that *N*-methylnipecotic acid was the predominant metabolite of arecoline or arecaidine (composing up to 30-38% of the dose). Such inconsistency could be partially explained by the interspecies differences in enzyme activity (e.g., reducing carbon-carbon double bonds of arecaidine to form *N*-methylnipecotic acid) between mouse and human.

In conclusion, this study describes a simple, rapid, and reliable LC-MS/MS method for direct determination of five major urinary metabolites of areca nut and tobacco

alkaloids. When combined with on-line SPE and isotope dilution, this method could allow for high-throughput analysis of urinary metabolites without compromising quality and validation criteria. Three urinary metabolites of areca nut alkaloids, namely, arecoline, arecaidine, and *N*-methylnipecotic acid, were firstly shown in the urines of regular chewers and were found to be highly correlated with the self-reported daily consumption of areca nuts. Good correlations may further illustrate the adequacy of the three metabolites as biomarkers for assessing exposure to areca nuts. Interestingly, however, our results of time course study showed that for arecoline, being the most abundant alkaloid in areca nut, very little was excreted unchanged (0.03-0.07% of the dose) with a short half-life of 0.97 hour after administration of areca nuts water extracts. This finding indicates that arecoline alone may not be a suitable biomarker and the information of interest may have been missed owing to its rapid elimination. Therefore, from the point of view of practical application, simultaneous determination of arecoline, arecaidine, and *N*-methylnipecotic acid (together composing 29-47% of the dose administered) could provide a more comprehensive evaluation of areca nuts exposure. Epidemiologic studies have shown that most areca nut chewers also have tobacco-chewing or cigarette-smoking habits. The combination of both habits has been shown to dramatically increase the risk of developing oral cancer up to ~90 times (40). Our method for determination of three metabolites of areca nut alkaloids together with nicotine and cotinine may facilitate the research into the oncogenic effects of both areca nut and tobacco exposure.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank the National Science Council, Taiwan, for financial support (Grants NSC 97-2314-B-040-017-MY2 and NSC 97-2314-B-040-015-MY3), and Chih-Ming Chen and Wei-Lin Cheng for help in sample preparation.

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Received 05/07/2010; revised 06/18/2010; accepted 07/06/2010; published OnlineFirst 09/14/2010.

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